

MACROMOLECULES AND INTERACTIONS AT THE CHONDROCYTE CELL SURFACE

Yngve Sommarin



Lund 1987



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In memory of my father

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LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals.

- I. Specific interaction between cartilage proteoglycans and hyaluronic acid at the chondrocyte cell surface.
Yngve Sommarin and Dick Heinegård.
Biochem. J. (1983) 214, 777-784
- II. Four classes of cell-associated proteoglycans in suspension cultures of articular-cartilage chondrocytes
Yngve Sommarin and Dick Heinegård
Biochem. J. (1986) 233, 809-818
- III. Metabolism of cell-associated proteoglycans in cultures of articular cartilage chondrocytes.
Yngve Sommarin and Dick Heinegård
In manuscript.
- IV. Two novel matrix proteins isolated from articular cartilage show wide distributions among connective tissues.
Dick Heinegård, Thomas Larsson, Yngve Sommarin, Ahnders Franzén, Mats Paulsson and Erik Hedbom
J. Biol. Chem. (1986) 261, 13866-13872
- V. Binding of isolated chondrocytes to extracellular matrix proteins from cartilage.
Yngve Sommarin, Thomas Larsson and Dick Heinegård
In manuscript.
- VI. Assay of proteoglycan populations using agarose-polyacrylamide gel electrophoresis.
Dick Heinegård, Yngve Sommarin, Erik Hedbom, Jörgen Wieslander and Bengt Larsson
Anal. Biochem. (1985) 151, 41-48

ABBREVIATIONS

CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate

CS - chondroitin sulphate

DS - dermatan sulphate

ELISA - enzyme-linked immunosorbent assay

HA - hyaluronic acid

HABr - hyaluronic acid binding region

HS - heparan sulphate

kDa - kilodalton

KS - keratan sulphate

PG-LA - large aggregating proteoglycan

PG-S - small proteoglycan

SDS - sodium dodecyl sulphate

INTRODUCTION

Connective tissues have abundant extracellular matrices surrounding the few cells. Cartilage represents an extreme where the cells occupy only about 2% of the tissue volume. The important functional properties of cartilage, i.e. its ability to distribute mechanical load, its elasticity and resistance to compression are the result of the properties of individual components of the extracellular matrix and their interactions. The chondrocyte is, however, of central importance for the function of cartilage in that it synthesizes the components and directs their assembly into an ordered matrix. The cell apparently is able to sense and regulate the composition of the matrix in response to changing demands. This ability is necessary in order to maintain the functional properties of the matrix during a life time.

Much is known about biosynthesis and assembly of the dominating components of the matrix, but knowledge is scarce on how the chondrocyte senses and regulates the composition of the matrix by concerted synthesis of several matrix components. This work is therefore focused on studies of the interplay between the chondrocyte and matrix constituents.

THE ENVIRONMENT OF THE CHONDROCYTE

Anatomical considerations

Three kinds of cartilage, hyaline, fibrous and elastic, can be distinguished on the basis of gross anatomical appearance. Hyaline cartilage, which is that most abundant, is in the adult found on the articular surfaces of joints, in nasal septum, larynx and trachea as well as in the junctions between ribs and sternum. During foetal development the skeleton is first formed as a precursor of hyaline cartilage, which is later calcified and transformed into bone. It should be noted, however, that although different hyaline cartilages have similar morphological appearance they are of different developmental origin. Although all cartilages are of mesenchymal origin they are not all of mesodermal origin. In the head region, neural crest cells migrate during development and eventually develop into tracheal, laryngeal and nasal cartilages. Other types of cartilages have different compositions. Thus fibrous cartilage in the menisci of the knee and the anulus fibrosus of the intervertebral discs have

a high contents of collagen fibers. Only so called elastic cartilages, which forms the outer ear, the auditory tube and the epiglottis, contains elastin. It is therefore apparent that cartilage represents a heterogeneous group with different composition among its members. Even a given cartilage usually does not have a uniform appearance. Especially the structure of articular cartilage varies going from the surface to the underlying bone forming three distinct zones of uncalcified cartilage distinguished on the basis of the appearance of the chondrocytes and the extracellular matrix (Stockwell, 1979; Schenk et al, 1986). A fourth zone of calcified cartilage follows adjacent to the underlying bone. Other cartilages also show a regional structural and compositional variation. To further complicate the situation, the extracellular matrix of cartilage is of non-uniform composition comparing the cell surface environment to the extracellular matrix. At the ultrastructural level a thin layer of pericellular matrix can be seen close to the chondrocyte (Eggli et al., 1985). This is surrounded by a territorial matrix with thin irregularly oriented collagen fibers forming a basket structure around the cell at the interface to the next region of the matrix, the interterritorial matrix (see Poole et al., 1984). The bulk of the intercellular substance is found in the interterritorial matrix with well separated thin collagen fibers and large amounts of proteoglycans occupying the space between fibrils. A major problem encountered in a studies of the ultrastructure of cartilage by electron microscopical techniques is shrinkage of the cells during fixation (Hunziker et al, 1983). Also the appearance of the extracellular matrix constituents is severely affected by inappropriate techniques. Using traditional fixation, proteoglycans collapse to a granular appearance. Improved methodology for fixation is therefore essential for better representation of the matrix. One promising technique is the recently developed rapid freeze fixation and freeze-substitution that gives much better preservation of fine details in the matrix and also improve chondrocyte appearance (Akisaka & Shigenaga, 1983; Hunziker & Schenk, 1984; Hunziker et al., 1984).

Taken together the developmental and structural heterogeneity between and within cartilages emphasize the need to consider different cartilages as a diversified group of tissues with considerable structural differences but sharing a basic molecular composition.

Macromolecular composition of the extracellular matrix.

Hyaline cartilage contains mainly water (60-80% of wet weight), proteoglycans (20-40% of dry weight) and collagen (40-70% of dry weight). The remainder is composed of non-collagenous proteins, lipids, nutrients and inorganic material.

The important mechanical properties of cartilage can largely be accounted for by the content and organization of water, proteoglycans and fibrillar collagen. The functions of the minor macromolecular components are not clear but it is likely that they are essential for the regulation of synthesis of the major components and their assembly. Indeed, the function of one of the minor cartilage components, the link protein, is to stabilize the assembly of proteoglycans into large aggregates in the extracellular matrix.

Proteoglycans

Proteoglycans consist of a central core protein to which a variable number of glycosaminoglycan chains are attached. Most proteoglycans also appear to be substituted with oligosaccharides. Variations in the number, size and type of glycosaminoglycan chains, oligosaccharides, size of core protein and regional distribution of substituents on the core protein give rise to a remarkable degree of structural complexity of proteoglycans. The description below is largely limited to matrix proteoglycan of cartilage and cell associated proteoglycans from other tissues.

Large cartilage proteoglycans. The proteoglycans considered typical for cartilage have a large core protein with reported molecular weights of 200 000 to 360 000 Da with large variations depending on the source, enzyme modifications to remove side chain substituents and method for determining molecular weight (Hascall & Riolo, 1972; Treadwell et al., 1980; Kimura et al., 1981; Olson et al., 1985). The core protein constitutes only about 8% of the mass of these proteoglycans. The bulk of the molecule is made up by 80-100 chondroitin sulphate chains (Hascall & Sajdera, 1970; Heinegård et al., 1985b) and about 50 keratan sulphate chains. The core protein is also substituted with some 50 O-glycosidically linked and 5-10 N-linked oligosaccharides (Heinegård et al., 1985b) such that the intact molecule has an M_r of 1.3×10^6 .

Three distinct structural regions have been identified within the proteoglycan core protein. At the N-terminal end a region is devoid of glycosaminoglycans. This region, called the hyaluronic acid binding region (HABr), forms a structure promoting specific interaction with hyaluronic acid. Electron microscopy, using rotary shadowing, reveals the presence of two globular structures in this region whereof only the one most N-terminal appears to interact with hyaluronic acid (Wiedemann et al., 1984; Paulsson et al., to be published). In the direction towards the C-terminus the HABr is followed by a region where most of the keratan sulphate chains are bound. The chondroitin sulphate attachment region then represents the major portion of the remainder of the molecule towards the C-terminus. A portion of the keratan sulphate chains of the proteoglycan are found in this region (Heinegård & Axelsson, 1977). Its major substituents, i.e. the chondroitin sulphate chains, are arranged in clusters of up to ten chains with regions of unsubstituted core protein between the clusters (Heinegård & Hascall, 1974b).

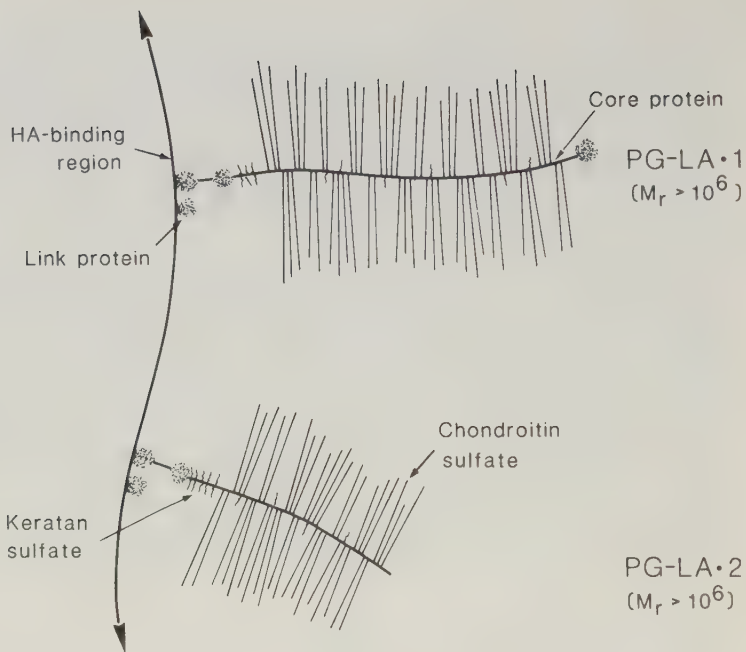
Recently the presence of a fourth region, a globular structure located at the C-terminus, was shown (Paulsson et al., unpublished). This region, of about 20kDa, carries no glycosaminoglycan chains has also been identified by partial cDNA sequencing of core proteins from rat chondrosarcoma (Doege et al, 1986), chick sternal (Sai et al., 1986) and bovine articular cartilage (Oldberg et al., 1986). The sequence shows a great deal of identity, i.e. more than 90% of the residues are the same indicating important functional properties also of this region. It appears that this region is cleaved from the PG by partial proteolysis, since this globular structure is absent on the majority of the PG molecules isolated from cartilage (Paulsson et al., to be published).

The ability of the core protein to bind to hyaluronic acid is fundamental for formation of very large aggregates containing up to 100 large proteoglycans bound to one hyaluronic acid molecule (Hardingham & Muir, 1972; Hascall & Heinegård, 1974; Rosenberg et al., 1975; Thyberg et al., 1975; Buckwalter & Rosenberg, 1983). By being bound in these large aggregates, proteoglycans are retained in the tissue. Although the binding of proteoglycan to hyaluronic acid is strong with a K_D of 10^{-8} - 10^{-7} (Christner et al., 1978; Cleland, 1979; Nieduszynski et al., 1980), it is further strengthened by a third molecule, the link protein. It binds to both

the proteoglycan core protein and the hyaluronic acid (Hardingham, 1979; Heinegård & Hascall, 1979a; Franzén et al., 1981; Tengblad, 1981) making the formation of the ternary complex practically irreversible under physiological conditions. The link proteins are present in purified aggregates in a molecular ratio to proteoglycan monomers of one (Heinegård & Hascall, 1974a; Kimura et al., 1980).

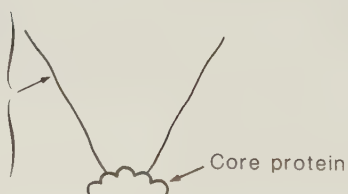
The formation of aggregates have been shown to occur after secretion from the chondrocytes (Kimura et al., 1979,1980; Björnsson & Heinegård, 1981). Interestingly, it has later been shown that hyaluronic acid is synthesized in the plasma membrane in studies of several cell types (Prehm, 1983 a,b; Philipson & Schwartz, 1984; Mian, 1986 a,b) and is continuously fed into the extracellular matrix during synthesis. However, it has not been shown whether proteoglycans and link proteins are exported separately or as a complex already formed intracellularly. A proteoglycan-link protein complex would probably form stable aggregates with hyaluronic acid directly at the cell surface. An explanation of aggregate formation, however, has to incorporate mechanisms for directed deposition of the very large aggregates that are less likely to diffuse from the cell surface to sites far away in the matrix. It may be important that newly synthesized proteoglycans exhibit lower affinity for hyaluronate than proteoglycans which have been present in the matrix for some time (Oegema, 1980; Bayliss et al., 1983; Plaas et al., 1983). This may result in delayed aggregation, potentially important for the formation of aggregates at distance from the cell. It appears, however, that proteoglycans still bind link protein and that this link protein can mediate binding of proteoglycan to hyaluronate.

Proteoglycans in addition to being structurally very complex, are very polydisperse, varying both with respect to size and charge. Studies of newly synthesized proteoglycans show that their core proteins are uniform in size but sizes of their side chains vary (Fellini et al., 1981). On the other hand, proteoglycans isolated from adult normal cartilage appear to have core proteins of variable size (Heinegård, 1977). It is likely that the polydispersity seen in preparations of proteoglycans is the result of depolymerization in the matrix. In support most cartilages contain comparatively high levels of "free HABr" (Roughley et al., 1985) which probably is the only structure of the proteoglycan retained in the tissue



CHONDROITIN SULFATE
Nasal cartilage

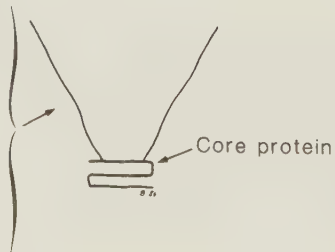
DERMATAN SULFATE
Articular cartilage
Aorta intima



PG-S.1
($M_r < 10^5$)

CHONDROITIN SULFATE
Adult bone

DERMATAN SULFATE
Articular cartilage
Tendon
Sclera
Cornea



PG-S.2
($M_r < 10^5$)

Figure 1. Schematic illustration of the main proteoglycan populations present in cartilage.

bound to hyaluronic acid after degradation of the glycosaminoglycan containing part of the core protein.

Preparations of large aggregating proteoglycans are not only polydisperse but consists of two apparently distinct subspecies of large proteoglycans. This has been apparent for a long time since agarose/polyacrylamide gel electrophoresis resolves preparations of large proteoglycans in two surprisingly sharp bands (McDevitt & Muir, 1971; Stanescu et al., 1977). Proteoglycans corresponding to each of the two bands have been isolated from nasal cartilage and characterized (Heinegård et al., 1985b). They differ in size and relative proportions of side chain substituents, fig. 1. It is not clear whether they represent distinct biosynthetic units or have a precursor-product relationship. In a study of ageing and proteoglycans from tracheal cartilage, only the larger subpopulation was found in young animals. During growth and maturation the relative content of the smaller subpopulation increased to reach a proportion in the adult animal that remained rather constant during subsequent ageing (Inerot & Heinegård, 1983). An indication of a precursor-product relationship was seen in a study of proteoglycan turnover in cultured human articular cartilage (Oegema & Thompson, 1986) where transformation of the larger proteoglycan into the smaller was seen. Similarly in vivo studies of proteoglycan metabolism in aged rats indicated a precursor-product relationship of the two proteoglycans (Heinegård et al., 1986).

Small cartilage proteoglycans. In addition to large proteoglycans, cartilage contains small amounts (less than 1% by weight) of low molecular weight proteoglycans with M_r of less than 100 000. This type of proteoglycan has been isolated and characterized from nasal and articular cartilage (Heinegård et al., 1981; Rosenberg et al., 1985). While the proteoglycan from nasal cartilage contains chondroitin sulphate chains, the molecules from articular cartilage has dermatan sulphate chains. The core proteins are of similar size, apparent M_r about 45000, but peptide pattern analysis and immunological cross-reactivity show that two distinctly different core proteins are present (Heinegård et al., 1985a; Hedbom, Heinegård & Rosenberg, unpublished). Small proteoglycans with only one type of core protein is present in nasal cartilage, whereas the two types of proteoglycans with different core proteins are found in articular cartilage.

One is apparently identical with the one from nasal cartilage and the other with small proteoglycans obtained from number of different sources such as tendon, sclera, cornea, bone and skin. Thus it appears that the core proteins of both types can be substituted with either chondroitin sulphate or dermatan sulphate chains, fig.1. The number of glycosaminoglycan chains on these proteoglycans may vary, but appears to be only one or two (Heinegård et al., 1981; Pearson et al., 1983). The function of these small proteoglycans is obscure but in a study of collagen fibril formation a small proteoglycan from tendon reduced the rate of fiber formation (Vogel et al., 1984). In support of an interaction with collagen fibers, small proteoglycans are located in a characteristic banding pattern on collagen fibers (Scott, 1984, 1986).

Cell-associated proteoglycans. Heparan sulphate proteoglycans appear to be present at all cell surfaces. Kraemer originally discovered the cell surface localization of a class of heparan sulphate probably in proteoglycan form (Kraemer, 1971). Since then, heparan sulphate proteoglycans have been characterized from rat liver plasma membranes (Oldberg et al., 1979). Two forms of these small proteoglycans, M_r about 100 000 for the intact molecule and core protein size of about 30000, have been identified. One is intercalated in the plasma membrane and the other is bound via a cell surface receptor recognizing the heparan sulphate side chains (Kjellén et al., 1980, 1981). Another interesting example of heparan sulphate proteoglycans are the two main types found in fibroblast cultures (Cöster et al., 1986). One has structural similarities with the transferrin receptor and its core protein consist of a disulphide bonded dimer intercalated in the plasma membrane and with an overall molecular weight of about 350000 (Cöster et al., 1983). This proteoglycan is found in confluent fibroblast cultures whereas the other smaller heparan sulphate proteoglycan that has a single core protein is prominent in growing fibroblasts (Cöster et al., 1986). It is found both as an intercalated form in the plasma membrane and in the matrix.

Not all cell associated proteoglycans contain heparan sulphate. A comparatively large proteoglycan, which appears to be a characteristic of melanoma cells, contains chondroitin sulphate as the only glycosaminoglycan (Bumol & Reisfeld, 1982; Ross et al., 1983; Bumol et al., 1984). Its core protein can be present on the cell surface in two forms,

either as an M_r 250000 glycoprotein or as a proteoglycan with chondroitin sulphate chains (Harper et al., 1986). Incubation of the melanoma cells with ammonium chloride inhibits synthesis of the proteoglycan form by interfering with intracellular processing. Proteoglycan biosynthesis has been well characterized in rat ovarian granulosa cell cultures (Yanagishita & Hascall, 1983, 1984 a,b). These cells synthesize one species of heparan sulphate proteoglycan and two classes of dermatan sulphate proteoglycans. The core protein appears to be the same for the heparan sulphate proteoglycan and the smaller of the dermatan sulphate proteoglycan. The large dermatan sulphate proteoglycan, which is quantitatively exported into the medium, has a core protein of approximately the same size as the melanoma cell proteoglycan (Yanagishita & Hascall, 1983).

The large cartilage proteoglycans have for long time been the only proteoglycans known which are substituted with two types of glycosaminoglycan on the same core proteins, i.e. both chondroitin sulphate and keratan sulphate. Recently, heparan sulphate-chondroitin sulphate containing proteoglycans from mouse mammary epithelial cells have been described (David & Van der Bergh, 1986; Rapraeger et al., 1986).

Intracellular proteoglycans. These proteoglycans are apparently located in storage granules of certain cells. Heparin proteoglycans have been isolated from mast cell tumours (Robinson et al., 1978) and secretory granules of mouse mast cells (Razin et al., 1982; Stevens et al., 1985) The latter may alternatively contain an over-sulphated chondroitin sulphate proteoglycan probably having the same protein core (Stevens, 1986).

When studying cell-associated proteoglycans, one should also consider the intracellular degradation pools present as exemplified in the study of Yanagishita and Hascall, 1984b.

Collagen of cartilage

An important function of collagen in cartilage is to form a fibrous network giving high tensile strength to the tissue. Nearly 90% of the collagen in cartilage is type II (Mayne & von der Mark, 1983), which forms relatively thin collagen fibers in the interterritorial matrix. Cartilage also contains a number of minor collagens: types V, VI, IX and XI (formerly

called 1 α 2 α 3 α) (for reference see Mayne & Irwin, 1986) each contributing 1-2% of the total collagen content (David Eyre, personal communication).

Type V and XI, together with type II, belong to the fibril forming collagens (Miller, 1985). It appears that in some tissues type V collagen may be copolymerized with type I collagen fibrils (Linsenmeyer et al., 1985). In analogy type XI may be a copolymer with type II fibrils.

Type IX collagen, which belongs to the short helix collagens, was originally isolated from chick embryo cartilage (Noro et al., 1983) as a low buoyant density chondroitin sulphate proteoglycan and later shown to be identical with type IX collagen (Vaughan et al 1985). The glycosaminoglycan chain is bound to the α 2(IX) chain (Bruckner et al., 1985). Type IX is codistributed throughout the matrix with type II collagen fibers and is possibly located at their intersections (Muller-Glauser et al 1986).

Type X collagen has a more narrow tissue distribution and appears to be unique for hypertrophic chondrocytes in growth cartilage (Schmid & Conrad, 1982; Schmid & Linsenmeyer, 1985).

Non-collagenous proteins of cartilage

Research on the macromolecular components of cartilage have almost exclusively concentrated on collagen and proteoglycans. Studies of the non-collagen proteins present in cartilage have been limited. Interestingly extracts of cartilage contains only a few distinct proteins (Paulsson & Heinegård, 1981). Therefore it is a feasible task to isolate and study function of most of these proteins. During recent years the non-collagenous proteins from cartilage have thus received increased attention. Today several matrix proteins have been isolated, but knowledge of their functions is in most cases scarce.

Link-protein. The best known non-collagenous protein of cartilage are the link-protein. Early observations showed the presence of two proteins codistributing with proteoglycans in isopycnic density gradients (Keiser et al., 1972). These proteins were shown to be components of proteoglycan aggregates (Heinegård & Hascall, 1974a).

Originally two forms were identified (Bonnet et al., 1978; Baker & Caterson, 1979). Recently it has become evident that several more forms are present either representing distinct biosynthetic products (Caterson et al., 1985; McKeown-Longo et al., 1983) or products of partial degradation (Caterson et al., 1985; Mort et al., 1985).

As described above link protein form a ternary complex with aggregating proteoglycan and hyaluronic acid in the extracellular matrix. The details of the assembly process is not entirely clear. Newly synthesized proteoglycans and also link protein are capable of binding to hyaluronic acid when secreted (Kimura et al., 1979; Björnsson & Heinegård, 1981). Thus the newly synthesized proteoglycan and link protein can form a functional stable ternary complex with hyaluronic acid added to the culture (Kimura et al., 1980) and endogenously formed aggregates appear to be immediately stable to competition with proteoglycan monomer (Björnsson & Heinegård, 1981). These observations suggest that a proteoglycan-link protein complex is formed before binding to hyaluronic acid (Kimura et al., 1980). As discussed above, however, binding of proteoglycan to hyaluronic acid is of somewhat lower affinity in newly synthesized molecules.

The 148kDa-protein. This protein was first identified as a component bound to proteoglycans from tracheal cartilage (Paulsson & Heinegård, 1979). It is a trimeric protein (Paulsson & Heinegård, 1981), but distinct from collagen. It is unique for cartilage and is most abundant in tracheal cartilage, whereas it has not been detected in articular cartilage (Paulsson & Heinegård, 1982). This emphasizes that there are indeed differences in matrix composition even within the group of hyaline cartilages. This may reflect developmental or functional differences. The amount of 148kDa protein also shows a marked age variation, being low in tracheas of young cows, reaching a maximum at 8 years of age and thereafter decreasing (Paulsson et al., 1984). In comparison, link protein showed small differences with age. The nature of the interaction between the 148kDa protein and large aggregating proteoglycans has not been clarified. Data indicate that binding to proteoglycan is via one of the three subunits with an as yet unknown covalent linkage (Hedbom, Paulsson & Heinegård, unpublished).

The 58kDa and 59kDa-proteins. Two distinct proteins of approximately the same size are found in cartilage and most other

connective tissue (Paper IV, this thesis). These proteins are further discussed below.

Collagen binding protein. Recently a protein with the ability to bind to collagen type II was isolated from the Swarm rat chondrosarcoma (Chandrasekhar et al., 1986). Type II collagen fibers in cartilage are considerably more thin and slender compared to collagen fibers in other tissues. When formed in vitro, however, the fibers have considerably larger diameters. In combination with large proteoglycans the collagen binding protein may interfere with collagen fiber formation in vitro, such that fibers formed have a smaller diameter. This may suggest a regulatory effect on collagen fiber formation in the tissue. Link protein has been shown to have a similar effect (Chandrasekhar et al., 1984) and also fibronectin affects the rate of collagen fibril formation (Kleinman et al., 1981). It should be stressed, however, that fibril formation also is very sensitive to a number of other experimental parameters such as pH, ionic strength and the type of buffer used (Vogel et al., 1984).

Fibronectin. Fibronectin, which appears to be present in all tissues, was for some time not detected in cartilage. It was considered to be produced only when chondrocytes were deprived of their matrix. It has, however, been shown that fibronectin in low amounts is a normal constituent of articular cartilage (Wurster & Lust, 1982). It can also be isolated from tracheal cartilage and is synthesized by adult chondrocytes surrounded by an intact matrix in explant culture (Larsson & Heinegård, unpublished).

Chondronectin. Chondronectin was first identified in serum and shown to be able to mediate interaction of chondrocytes with type II collagen substrates (Hewitt et al., 1980). It is therefore different from fibronectin which does not show a pronounced specificity for different collagens. By the use of immunofluorescence microscopy, chondronectin has been demonstrated in the vicinity of chondrocytes in cartilage (Hewitt et al., 1982). Chondronectin was later shown not to mediate binding to collagen by itself, but requires the presence of chondroitin sulphate proteoglycans (Varner et al., 1986). It should be stressed that to date synthesis of chondronectin by chondrocytes has not been demonstrated. The nature of the molecule and its true activity remains under debate.

400kDa-protein. A large multimeric protein with subunits of about 100 kDa upon reduction have been observed in cartilage (Spitz Fife & Brandt, 1984; Heinegård et al., unpublished). The intact protein has a molecular weight of more than 400 kDa. By immunofluorescence microscopy it appears to be localized to cartilage matrix only (Franzén, Solursh & Heinegård, unpublished).

Anchorin CII. A protein capable of mediating interactions between chondrocytes and type II collagen is a membrane intercalated 34kDa-protein named anchorin CII (Mollenhauer et al., 1983). It is isolated from chicken cartilage and when inserted in liposomes it shows a high specificity for binding to type II collagen. It is located in the pericellular space as shown by immunofluorescence microscopy (Mollenhauer et al., 1984). Other mechanisms for attachment of chondrocytes to collagen, however, are also likely to be active (Mollenhauer et al., 1984). Thus, chondrocyte attachment to collagen could not be completely blocked by anti-anchorin CII antibodies.

36kDa-protein. Another protein of 36kDa is found in many cartilages (Paulsson & Heinegård, 1982). It is a prominent biosynthetic product of chondrocytes in organ culture of tracheal cartilage (Paulsson et al., 1983). The protein is difficult to study because of insolubility in low salt buffers necessitating the use of chaotropic solvents. It has, however, been purified and characterized (Heinegård, Sommarin, Hedbom, Larsson & Paulsson, manuscript in preparation). The protein is not detected in other connective tissues measured by ELISA assay. Its function is not known, but when coated onto plastic surfaces it mediates the attachment of chondrocytes and fibroblasts but not hepatocytes (paper V this thesis).

Collagen II C-propeptide. During foetal development a dimeric protein, which upon reduction yields 35kDa subunits, increases with age in bovine foetal epiphyseal cartilage (Choi et al., 1983). This protein, named chondrocalcin, has been suggested to participate in the calcification process of cartilage (Poole et al., 1984). Recently this protein was shown to be identical to the C-terminal propeptide of type II collagen (Van der Rest et al., 1986), cleaved from the procollagen molecule in the extracellular matrix during collagen fibril formation.

REGULATION ON CHONDROCYTE SYNTHESIS OF MATRIX MACROMOLECULES

The chondrocyte is a highly specialized cell that is responsible for the synthesis and maintenance of an extensive extracellular matrix consisting of a multitude of macromolecular components all cooperating to form a functional unit. Although we have considerable knowledge on biosynthesis and assembly of the basic units of the matrix, i.e. collagen fibers and proteoglycan aggregates, many essential questions regarding formation and maintenance of cartilage are not known. Where in the matrix does formation of fibrils and aggregates occur? How are chondrocytes able to sense defects in matrix composition? How does the chondrocyte respond to changing demands on the cartilage? How does the chondrocyte maintain the integrity of cartilage for so many years? What initiates the process and how does the chondrocyte respond during degenerative processes such as osteoarthritis?

Fundamental to the understanding of these problems is better information on the ability of the chondrocyte to interact with the matrix components therein and surrounding tissues. Indications on potential mechanisms have been obtained in studies of the influence of environmental factors, matrix components and hormones on chondrocyte synthesis and degradation of matrix components.

Modulation of the chondrocyte phenotype.

Several investigators have implicated a role of cell shape for expression of the chondrocyte phenotype (for review see Solursh, 1986; Benya & Brown, 1986). The traditionally used hallmark of the chondrocyte is its production of type II collagen and large aggregating chondroitin sulphate proteoglycans.

Culture of isolated chondrocytes under conditions which induce flattening of the cells eventually lead to loss of chondrocyte phenotype as is best documented as a changed collagen synthesis from type II collagen to type I and/or type I-trimer (Mayne et al., 1975; Benya et al., 1978). Culture conditions which promote preservation of a spherical cell shape results in retained collagen type II synthesis. Thus chondrocytes cultured on agar (Horwitz & Dorfman, 1973), in agarose (Benya & Shaffer, 1982), high

density monolayer (Kuettner et al., 1982), in monolayer with serum free medium on hydrophobic culture dishes (Björnsson & Heinegård, 1981) or in suspension culture (Deshmukh et al, 1976), retain their rounded shape and synthetic pattern.

The chondrocyte phenotype can also be altered by conditions like culture in the presence of bromodeoxyuridine (Mayne et al, 1975) or retinoic acid (Benya & Padilla, 1983).

Brief exposure of mesenchymal cells in limb development to the actin filament disrupting drug cytochalasin D will lead to rounding of the cells. This treatment also favours chondrogenic differentiation (Zanetti & Solursh, 1986). It appears that cytoskeletal elements have a role in the transmission of information on matrix properties to the cell.

The aggregating proteoglycans are often referred to as "cartilage specific", but more recently they have been demonstrated in small amounts in a number of other tissues including, aortic smooth muscle cells (Wight & Hascall, 1983), colon cells (Iozzo & Wight, 1982), glial cells (Norling et al., 1978, 1984), skin fibroblasts (Cöster et al., 1979) and tendon (Vogel & Heinegård, 1985) (see also Heinegård et al, 1985a). However, the chondrocyte produces much larger amounts of aggregating proteoglycan resulting in the formation of a prominent extracellular matrix. Synthesis of the large aggregating proteoglycans is decreased when chondrocytes are subcultured in monolayers. At the same time synthesis of small proteoglycans is increased (Srivastava et al., 1974; Madsen & Lohmander, 1979). This effect can be partly reversed in spinner culture. Effects on the glycosaminoglycan produced have also been reported (Oegema & Thompson, 1981) with a change from chondroitin sulphate to dermatan sulphate chains on large aggregating proteoglycans.

Synthesis of cartilage proteins is also affected by culture conditions. Thus expression of 148kDa protein is lost in culture of isolated chondrocytes (Heinegård et al., 1983). This can be prevented if chondrocytes are cultured with an intact surrounding matrix in explant cultures (Paulsson et al., 1983).

Modulation of the rate of proteoglycan synthesis.

Addition of matrix components to chondrocytes in culture have been shown to affect the rate of proteoglycan synthesis. Hyaluronic acid when added to isolated cells may inhibit proteoglycan production (Wiebkin & Muir, 1973; Handley & Lowther, 1976; Solursh et al, 1980) but the effect is not a consistent finding and seems to be dependent on culture conditions (Schwartz & Dorfman, 1975; Wiebkin & Muir, 1977). Proteoglycans on the other hand was first shown to increase the rate of proteoglycan synthesis (Nevo & Dorfman, 1972; Schwartz & Dorfman, 1975). The effect of proteoglycan addition seems to depend on the amount of proteoglycan added (Handley & Lowther, 1977; Lowther & Handley, 1979). Thus low amounts of added proteoglycan stimulate whereas large amounts (10 mg/ml) inhibit synthesis of proteoglycan. The mechanism for these effects is not known, but it is likely that it results from changing concentrations of matrix components at the cell surface. It is possible that addition of hyaluronic acid will lower the amount of proteoglycan at the cell surface by competition with cell surface hyaluronate for proteoglycan binding (Solursh et al., 1980; Sommarin & Heinegård, 1983, 1986) and that addition of proteoglycan may result in release of hyaluronic acid from the cell surface. However, the effects of hyaluronic acid on proteoglycan synthesis has a slow onset and can be obtained with hyaluronic acid tetrasaccharides whereas the displacement effect is rapid and requires hyaluronic acid oligomers that are decasaccharides or longer (Solursh et al., 1980). This indicates that receptors for hyaluronic acid may be present at the chondrocyte cell surface in analogy with receptors on fibroblast (Underhill & Toole, 1980) and liver endothelial cells (Laurent et al., 1986).

After culture of cartilage explants, the chondrocytes adapt with time and a new steady state situation is obtained where the loss of proteoglycan is equalled by synthesis (Hascall et al., 1983). This situation can be perturbed by proteolytic damage to the matrix whereafter the chondrocytes are able to restore the matrix composition by increased synthetic activity (Bartholomew et al., 1985).

Mechanical influences also appear to be important. Hydrostatic load applied to chondrocytes in culture have been shown to increase the rate of proteoglycan synthesis (van Kampen et al., 1985). On the other hand, relieving the load on articular cartilage will lead to pronounced loss of

proteoglycan from the cartilage (Palmoski et al., 1979, 1980). Lost proteoglycans are replaced when normal load is resumed.

Modulation of matrix turnover.

The activity of the chondrocyte is also affected by agents released from surrounding cells. This is exemplified in studies of the inflammatory process in joints in osteoarthritis. Explant cultures, where the chondrocyte is surrounded by an intact matrix, provided interesting information on regulation of matrix turnover with major focus, on proteoglycans. Thus, in studies of effects of various agents on cartilage matrix degradative in osteoarthritis, coculture of cartilage explants with synovial membrane biopsies has been useful (Steinberg et al., 1979). The effect of one of the products of macrophages in the synovial membrane, i.e. interleukin-1 also called catabolin on proteoglycan turnover, have been studied in detail (Saklatvala et al., 1984). It inhibits proteoglycan synthesis (Tyler, 1985b) and increases the normal proteoglycan degradation and release from the cartilage (Tyler, 1985a; Ratcliffe et al., 1986).

Other agents such as retinol (Jubb & Fell, 1980) and bacterial lipopolysaccharides (Morales et al., 1984) also induce degradation of matrix constituents and resorption of the cartilage.

PRESENT INVESTIGATION

The chondrocyte is embedded in an abundant extracellular matrix which is heterogeneous in appearance and composition. A thin pericellular zone surrounds the cells and is in turn surrounded by a territorial and an interterritorial matrix. At present little is known of the role of these regions and how deposition of matrix components in these compartments is regulated. Also information of the interaction of the cell with its matrix is scarce. However, an important region in the control of matrix, repair and remodelling should be the region of direct contact with the matrix. A better understanding of events at the cell surface is therefore likely to be an essential component in the understanding of cartilage biology.

In the present investigation the structures of and interactions at the cell surface of chondrocytes in culture are studied. Two questions are addressed: 1) Is the pericellular material identical in proteoglycan composition to the matrix (medium) or should it be considered a distinct

compartment with different properties? 2) Can interactions between the chondrocyte and distinct matrix components be identified?

Cell-associated proteoglycans of the chondrocyte (Papers I-III).

The aim of these investigations was to study the relationship between proteoglycans at the chondrocyte cell surface and proteoglycans of the matrix. A possible mechanism for interaction between proteoglycans and the chondrocyte was characterized. The nature of the proteoglycan composition at the cell surface and the turnover of these proteoglycans were studied.

Interaction between large aggregating cartilage proteoglycans and the chondrocyte cell surface (Paper I).

Proteoglycan aggregate formation has been shown to be an extracellular event using chondrocyte cultures (Kimura et al., 1979, 1980; Björnsson & Heinegård, 1981). However, in these studies, even with the cells kept in suspension, a substantial portion of the proteoglycans synthesized are associated with the cell surface. This raises the question whether these proteoglycans are bound to hyaluronic acid at the cell surface, perhaps forming cell-bound aggregates. Interestingly addition of proteoglycan or hyaluronic acid to cultured chondrocytes cause the cells to change their rate of proteoglycan synthesis (Nevo & Dorfman, 1972; Wiebkin & Muir, 1973; Lowther & Handley, 1979) indicating alterations of the cell surface, most likely involving interaction of proteoglycan with cell surface components.

The aim of this study was to examine possible mechanisms for binding of large aggregating proteoglycan to the chondrocyte. The experimental system chosen were articular cartilage chondrocytes kept in suspension culture. As a probe radiolabelled, large aggregating proteoglycans were prepared from culture medium and showed to be functional in being able to form aggregates with hyaluronic acid.

The labelled proteoglycans bound to the chondrocytes with a maximum within 2-3 hours. The binding could be saturated by increasing the amount of added labelled proteoglycan, indicating a limited number of binding sites. Bound proteoglycans could at all times be removed by trypsin digestion showing that at most a minor fraction was internalized.

Further studies of the mechanism for binding showed that hyaluronic acid is present at the cell surface and can act as a receptor for aggregating proteoglycans at the cell surface. This was corroborated in experiments with *Streptomyces* hyaluronidase digestion of cells with bound proteoglycan. Almost complete removal of bound labelled proteoglycan was obtained.

The binding of labelled proteoglycan to hyaluronic acid at the cell surface was not freely reversible indicating that binding was stabilized by an additional factor. In analogy with aggregate formation in the medium this stabilization may result from added endogenous link protein.

These observations demonstrate a mechanism for an interaction of matrix proteoglycan with the chondrocyte cell surface. They also point to the fact that proteoglycan aggregation may also take place in close proximity to the cell surface of chondrocytes. In a study by Goldberg and Toole (1984) hyaluronic acid was shown to be essential for retaining proteoglycans at the cell surface.

The mechanism for binding of hyaluronic acid to the cell surface of chondrocytes is not known. The most likely mechanism is binding to the hyaluronate synthetase in plasma membrane in analogy with hyaluronic acid synthesis in teratocarcinoma cells (Prehm, 1983a,b). In chondrosarcoma cultures hyaluronic acid accumulate in the medium with little retention in the cell layer (Mason et al., 1982). However, alternative mechanisms may be present since the study by Goldberg and Toole (1984) showed that high amounts of hyaluronic acid are retained at the cell surface. Binding experiments with added radiolabelled hyaluronic acid to our chondrocyte cultures have been negative (unpublished).

Binding of newly synthesized proteoglycans and characterization of cell-associated proteoglycans (Paper II).

The observations in paper I were extended in experiments studying binding of endogenous, newly synthesized proteoglycans to the cell as a complement to the previous experiments with added exogenous proteoglycan. Chondrocytes were cultured in medium containing [³⁵S]-sulphate to radiolabel newly synthesized proteoglycans.

Even with added large amounts of hyaluronic acid or large proteoglycans a proportion (about 50%) of the large proteoglycans remained associated with the cell surface. These cell surface proteoglycans were either not accessible for competition or they were bound by other mechanisms. This prompted the characterization of the cell-associated proteoglycan. A further goal was to establish if the composition of proteoglycans at the cell surface differed from the composition normally found in the medium or in the cartilage matrix.

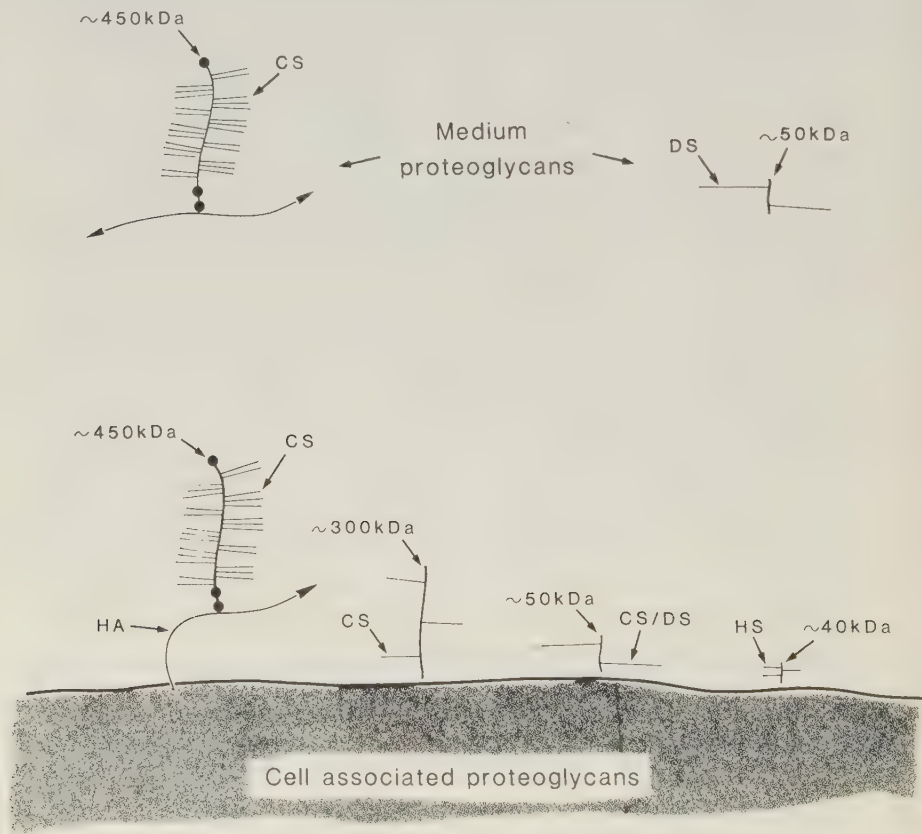


Figure 2. Schematic illustration of proteoglycans in the chondrocyte cultures.

The predominant proteoglycan produced in the cell cultures are the large aggregating proteoglycan found in the medium, fig. 2. It represents about 85% of the total labeled macromolecules in cultures with added hyaluronic acid. A similar type of proteoglycan is also found as a major component at the cell surface. Expectedly, this proteoglycan appears to be bound to hyaluronic acid, since competition with hyaluronic acid which reduces the amount of the proteoglycan at the cell surface substantially. Digestion of cells with *Streptomyces* hyaluronidase (taken proper precautions against proteolysis) further reduces the amount of this proteoglycan leaving only a small fraction associated with the cell. This was the only proteoglycan at the cell surface which appeared to be bound to hyaluronic acid. Further structural analysis indicated identity of the cell surface bound large proteoglycans with that in the medium.

An unexpected finding was the presence of a chondroitin sulphate proteoglycan of intermediate size which has not been detected in extracts of cartilage. This proteoglycan has a comparatively large core protein, with an apparent M_r of 300 kDa determined by SDS polyacrylamide gel electrophoresis. The proteoglycan is of very low buoyant density indicating substitution with only few chondroitin sulphate chains. Two other previously described proteoglycans from melanoma cells (Bumol et al., 1984) and from rat ovarian granulosa-cell culture (Yanagishita & Hascall, 1983) are of similar character.

Two additional smaller proteoglycans with dermatan sulphate and heparan sulphate side chains, respectively, were isolated from the cell surface. They could neither be separated by gel chromatography nor by ion exchange chromatography. The larger belongs to the class of small proteoglycans with core proteins of apparent M_r 45 to 50 kDa (Heinegård et al., 1985a). Proteoglycans of this type were also detected in the medium. However, the cell-associated and medium small proteoglycans were not identical. Thus the medium proteoglycan contained, dermatan sulphate chains with a higher degree of epimerization than those on the cell-associated proteoglycan. Close inspection of core proteins on SDS polyacrylamide gel electrophoresis revealed a heterogeneity of cell-associated small proteoglycans whereas the core protein of small proteoglycans in the medium appear homogeneous. It is possible that the two types of small dermatan sulphate proteoglycans synthesized by

articular cartilage chondrocytes correspond to the two subclasses of dermatan sulphate proteoglycans demonstrated in extracts of articular cartilage (Rosenberg et al., 1985; Hedbom, Heinegård & Rosenberg, unpublished).

The heparan sulphate proteoglycan detected was somewhat smaller in size than the dermatan sulphate proteoglycan at the cell surface. It had short heparan sulphate chains and a core protein of approximately 40 kDa determined by SDS polyacrylamide gel electrophoresis.

It is apparent that articular cartilage chondrocytes synthesize two species of proteoglycans not identified in cartilage extracts. These proteoglycans are found only associated with the cells and therefore may form part of the pericellular matrix surrounding chondrocytes in the cartilage.

Since articular cartilage chondrocytes differ with depth from the surface it is possible that cells in different layers express different populations of proteoglycans and that this is reflected in the diversity of proteoglycans produced in the cultures. Preliminary investigations of proteoglycans from chondrocytes of tracheal cartilage which appear to be more uniform, however, similarly indicate the presence of the various types of proteoglycans.

Release of proteoglycan aggregates from the cell surface.

The kinetics of proteoglycan release and binding back to the chondrocytes shown in fig. 1b (paper II) indicates that the proteoglycans bound to the cell surface are later again released into the medium. This suggests that the proteoglycans bound to hyaluronic acid at the cell surface may be released into the medium as aggregates. In a separate set of experiments we therefore studied whether large aggregating proteoglycans bound to hyaluronic acid at the cell surface actually can be released as intact aggregates (Sommarin & Heinegård, unpublished work).

The labelling protocol was designed to favour a situation where all large proteoglycans present at the cell surface would be bound to hyaluronic acid. The data in paper II show that [^{35}S]-sulphate labelled proteoglycans were exported into the medium within 10 minutes. Binding of exported large proteoglycans back to the cell was efficiently blocked

when large amounts of hyaluronic acid or proteoglycan monomer was included in the medium (fig. 1c, in paper II). In the present experiment, the cells were labelled with [^{35}S]-sulphate for 2 hours and a chase in non-radioactive medium was performed for 15 minutes to allow export of all free large proteoglycan monomers to the medium. This was replaced with fresh chase medium with or without a high concentration of large proteoglycan monomer, the latter preventing endogenous proteoglycan monomers from subsequently binding to hyaluronic acid. In the set of experiments with added proteoglycan monomers, also the first chase contained added proteoglycans.

Proteoglycans released into the medium during indicated times were directly analyzed by sucrose density gradient centrifugation to separate aggregates from monomers and small proteoglycans, fig. 3. Aggregates in cultures with added proteoglycan monomer, fig. 3b, were much larger than those without added proteoglycan and sedimented to the bottom of the gradient. This probably results from exogenous proteoglycans being bound to preformed aggregates where the hyaluronic acid was only partially substituted. In cultures without additions the detectable amount of aggregate was initially low, fig. 4a. Already at the initial time (20 min) of the chase with added monomer, a large proportion of aggregate was identified in the medium showing that stable aggregates could indeed be released from the cells and increase with time. The amount of aggregate released at early times in cultures without added proteoglycans was much less, indicating that when more proteoglycan was bound to the hyaluronic acid release from the cells was facilitated. At all times both media with and without added proteoglycan contained molecules sedimenting as free monomers. Whether these actually represent molecules capable of binding to hyaluronic acid or already bound to fragments of hyaluronate have not been studied due to lack of material.

Metabolism of cell-associated proteoglycans (Paper III).

The metabolic fate of the various proteoglycans identified at the cell surface of cultured chondrocytes was studied. Main focus was on whether the proteoglycans were released into the medium, i.e. in cartilage corresponding to release and incorporation into the matrix, or retained with the cells.

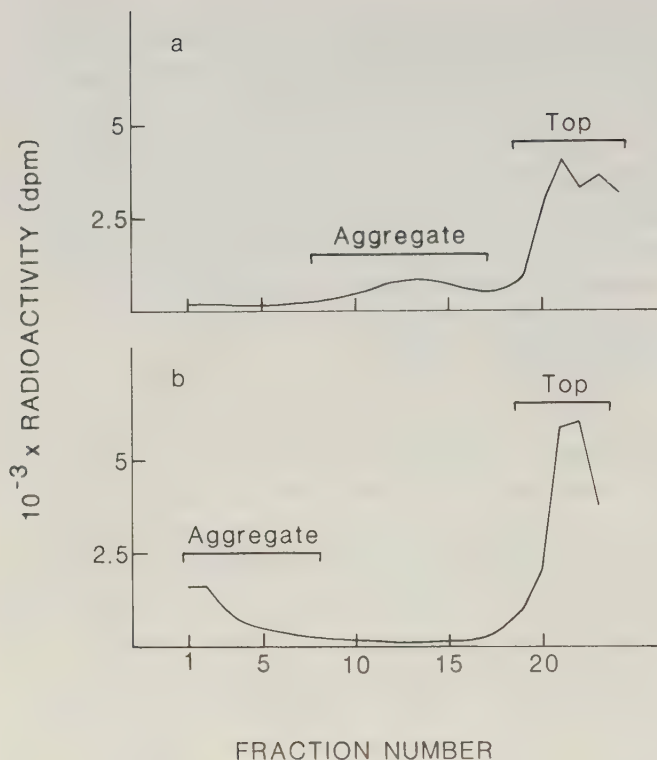


Figure 3. Sucrose density gradient separations of aggregates and monomers released into the medium.

Samples (200 μ l) of the second chase medium were layered on preformed sucrose gradients (10-50%) in 10 mM Tris-HCl pH 7.4 with 50 μ g bovine serum albumin/ml. Gradients were centrifuged for 50 min at 240000 g, 20°C in a Beckman SW60 swing out rotor. Centrifuge tubes were emptied from the bottom with the aid of a peristaltic pump as described elsewhere (Franzén et al., 1982) and fractions of 160 μ l were collected; 50 μ l/fraction were taken for determination of radioactivity.

Under these conditions proteoglycan aggregates were rapidly separated from nonaggregated large proteoglycan, small proteoglycans and free isotope which remain at the top of the gradient. The examples shown are separations of 40 min chase media.

a) No additions. b) Bovine nasal cartilage proteoglycan monomer (3 mg/ml) included during chase.

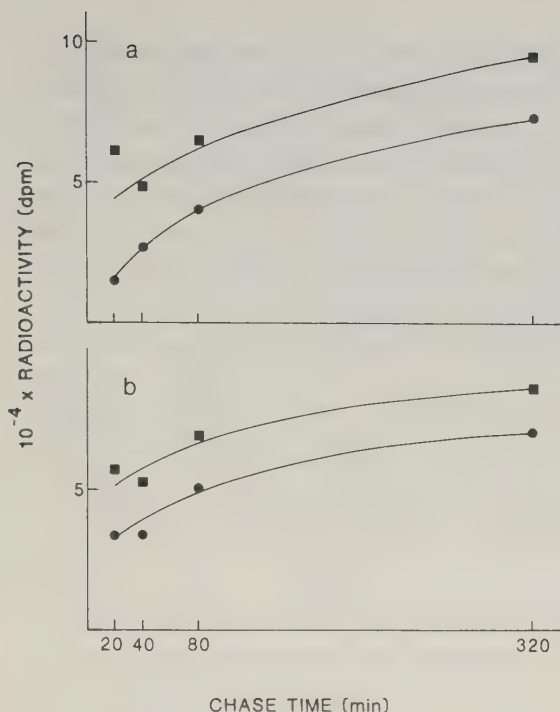


Figure 4. Release to the medium of proteoglycan aggregates and monomers.

The amount of macromolecular [^{35}S]-sulphate (proteoglycan monomer plus small proteoglycans) in top fractions of the sucrose gradients was determined by gel permeation chromatography on Sephadex G50. The amount of proteoglycan monomer in the medium, i.e. aggregated as well as not aggregated, was determined by chromatography on Sephacryl S-500 of the medium (data not shown). Combining these figures, the amounts of labelled proteoglycan aggregate and monomer respectively were calculated. proteoglycan was obtained from the gradients and the amount of large proteoglycans, i.e. both aggregate and monomer, to small proteoglycans was obtained from gel permeation chromatography on Sephacryl S500. From these data the amounts of labelled proteoglycan aggregates and monomer were calculated.

a) No addition. b) Bovine nasal cartilage proteoglycan monomer included during chase.

Aggregates (filled circles). Monomers (filled quadrants).

In the experiments described in paper II, the proteoglycans studied were synthesized during a four hour labelling period. Preliminary studies indicated a slow removal of particularly the intermediate size proteoglycan from the cell surface. To study proteoglycan turnover during a longer time period the culture conditions had to be modified, since the cells only retain activity for some 6 hours in high density suspension culture. Chondrocytes were therefore plated and cultured on hydrophobic plastic plates. In the absence of serum the cells attach, but do not spread, and retain their synthetic activity (Björnsson & Heinegård, 1981a). The cells do under these culture conditions not deposit large amounts of pericellular proteoglycans. In control experiments it was shown that the cell associated proteoglycans gave chromatographic elution patterns essentially identical to those obtained with cells in suspension, with presence of all four types of proteoglycans. Thus, also the the monolayer culture is well suited for studies of turnover of proteoglycans at the cell surface.

The large proteoglycans predominate in the cultures, both at the cell surface and in the medium. Gel permeation chromatography used for separations of proteoglycan populations unfortunately only provides partial separation precluding quantitations. A CsCl-centrifugation step was therefore introduced for the separation of the large, high buoyant density proteoglycans from the low buoyant density proteoglycans of intermediate and small size. Good separation of the various classes was then obtained by gel permeation chromatography.

Elimination of proteoglycans was studied after prelabelling of the molecules with [^{35}S]-sulphate. All the cell-associated proteoglycans showed a biphasic elimination from the cell surface. A rapid phase, with half lifes of proteoglycans ranging from 4 to 8 hours was apparent during the first one to two days. Later during culture the slow phase became apparent, with half lifes of the large proteoglycans of about 4 days. The corresponding half life for the intermediate size proteoglycans as well as for the small proteoglycans was about 8 days. The dermatan sulphate and the heparan sulphate small proteoglycans could not be studied separately, but the data indicated a more rapid elimination of the heparan sulphate proteoglycan.

The data showed that the large proteoglycan was quantitatively released to the medium. A large proportion of the intermediate size proteoglycan

also released from the cells could be identified in the medium, while only a minor proportion of the small proteoglycans could with certainty be recovered in the medium. It thus appears that the intermediate size proteoglycan may represent a novel matrix constituent that has previously been overlooked, possibly because of its low buoyant density.

Proteoglycans of similar nature have previously been identified in cultures of rat ovarian granulosa cells, where it is released to the medium (Yanagishita & Hascall, 1984b), and cultures of melanoma cells where it is mostly associated with the cell surface (Bumol et al., 1984).

The mechanism for release of the proteoglycans from the cell surface is at present not clear. The size distributions of those molecules released to the medium, however, although essentially showing the same size of the molecules indicated a more pronounced trailing, which may point at limited proteolytic cleavage.

Interaction of the chondrocyte with matrix proteins (Papers IV-V).

Several less predominant matrix components in addition to the major products, collagen and proteoglycans, are synthesized by chondrocytes. The function of these are for the most part not known. It is reasonable to assume that some of these molecules could affect the activities of the chondrocyte. An interaction of the particular molecule with the cell is likely to be involved in such a process.

As a part of a long term program we have isolated and characterized several of the major non-collagenous proteins of cartilage. One goal is to improve our understanding of matrix organisation and properties. Other goals with this approach is to use the proteins as markers of remodelling and repair of cartilage. Thus two distinct proteins have been isolated from articular cartilage. These were then tested, as well as other matrix proteins, for ability to adhere chondrocyte as indication of cell interactions with matrix component.

Isolation, characterization and distribution of two matrix proteins present in articular cartilage (Paper IV).

A major band seen on SDS gel electrophoresis of extracts of articular cartilage is a non-disulphide bonded protein component of about 60 kDa (Paulsson & Heinegård, 1982). This component was purified by a

combination of CsCl density gradient centrifugation and gel chromatography. Upon ion exchange chromatography on DEAE-cellulose the material separated into two main proteins. One did not bind to the ion exchange resin whereas the other required fairly high salt concentrations for elution, showing its anionic character. The protein which did not bind to the ion exchanger had an M_r of 58 kDa and the bound protein had an M_r of 59 kDa. Both proteins had high contents of leucine and aspartic acid/asparagine, a feature characteristic for several matrix molecules. However, the proteins are not related to each other as shown by different tryptic peptide patterns and the lack of cross-reactivity in radioimmunoassays developed for each of the two proteins.

The radioimmunoassays were used to study the distribution of the proteins in a number of tissues. Both proteins could be detected in all cartilages examined but with different amounts ranging from 0.1% to 0.3% of tissue wet weight. Most other connective tissues tested contained detectable amounts, with sclera and tendon containing larger amounts than the other non-cartilage tissues. Only the 59 kDa protein could be detected in serum.

Immunofluorescence microscopy was used to study distribution of the two proteins within cartilage. Most intense staining was obtained in the territorial matrix near the cells.

Chondrocyte interaction with matrix proteins (Paper V).

A major problem studying matrix proteins of cartilage is the insolubility of several proteins in low ionic strength buffers, typically noted for the 148 kDa proteins, link proteins, the 36 kDa protein and the 400 kDa protein. This precludes studies of binding to cells using the classical approach with radiolabelled molecules added to the culture medium. An alternative approach, therefore, is to use a solid phase binding assay such as coating of plastic surfaces with the protein of interest and measuring the ability of cells to attach to the coated surface. An added advantage of this assay is that interaction due to weak multiple binding can be detected such as attachment of cells to fibronectin.

With this approach binding of chondrocytes to the 36 kDa and the 58kDa protein could be demonstrated. The 36 kDa protein showed

consistently good attachment of cells, equal to that of fibronectin and type II collagen. Attachment to 58 kDa protein varied somewhat between experiments. This may indicate that an additional factor may be involved in 58 kDa-protein mediated attachment. This is not a serum factor since all experiments were performed without added serum.

Both proteins could interact with other loose connective tissue cells such as sclera fibroblasts. Hepatocytes on the other hand did not attach to the 36kDa and 58kDa proteins, while expectedly attachment to fibronectin was efficient. This indicates that binding of chondrocytes to the two proteins probably results from an interaction with molecules only present at surfaces of some cell types.

The mechanisms for binding to these proteins were not established. It is not related to the class of arginine-glycine-asparagine (RGD) specific receptors for matrix components (Ruoslahti et al., 1985) since competition with RGD containing peptides did not prevent attachment.

One interesting aspect is whether binding is directly to receptors at the cell surface or to molecules in the pericellular matrix. Usually the chondrocytes were kept in spinner culture for 1-2 days before use to allow reconstitution of cell surface components after the harsh enzymatic treatment used to liberate the cells from the extracellular matrix. During this period the cells were given the opportunity to assemble a pericellular matrix which perhaps shield some binding sites. Additional experiments thus showed that chondrocytes used directly after cell isolation bound to the proteins discussed above, but also to surfaces coated with large or small proteoglycan. The results indicate alterations in the pericellular coat of the cells during spinner culture. These effects were not further studied.

Development of composite agarose/polyacrylamide gel electrophoresis (Paper VI).

With the increasing awareness of the the presence of different populations of proteoglycans the need for high resolution analytical methods has increased. One such method is composite agarose/polyacrylamide gel electrophoresis which already 1971 was applied to the study of large cartilage proteoglycans by McDevitt and Muir. They found two sharp bands in preparations of proteoglycans apparently

homogeneous in studies with other techniques. The significance of this result was not clarified until recently when different populations of large proteoglycans corresponding to each of the two bands were isolated and characterized (Heinegård et al., 1985b). In addition to the two major bands, a faster migrating third band was observed. It corresponds to the class of small proteoglycans first studied by agarose/polyacrylamide gel electrophoresis by Stanescu and coworkers (1981). The intermediate size proteoglycan identified at the cell surface of chondrocytes show a characteristic slow migration (Paper II). The method can also be used to analyze differences in substructures of large aggregating proteoglycans (Heinegård et al., 1985b).

A major disadvantage of the original method was the need for highly purified preparations of proteoglycans. In the present development the method have been adapted to allow analysis of crude tissue extracts by the use of detergent treatment of samples. It is also shown that the staining procedure can be used to quantitate components manner. An adaptation of the system to measure aggregation of proteoglycans with hyaluronic acid is also presented. This procedure has, compared with conventional analysis of aggregation by gel chromatography, the advantage of also detecting weak binding of proteoglycans to hyaluronic acid.

Recently similar adaptations of agarose/polyacrylamide gel electrophoresis have been made (Carney et al., 1986; Kimura et al., 1986).

CONCLUDING REMARKS

In the present work the chondrocyte cell surface is studied in terms of interactions with proteoglycans and matrix proteins. Large aggregating proteoglycans were shown bind to hyaluronic acid at the cell surface. All proteoglycans at the cell surface were however not bound to hyaluronic acid.

Investigations of proteoglycans at the cell surface of cultured chondrocytes revealed the presence of proteoglycans previously not known in cartilage. Metabolic data indicate that the intermediate size, low buoyant density proteoglycan described, may be found in cartilage matrix. This possibility is presently being investigated. If successful, specific antibodies to this proteoglycan can be raised and the distribution between tissues and

localization of this proteoglycan can be determined, a basis for the understanding of its function.

A number of matrix proteins were tested for cell-binding activity. One of these proteins, the 36kDa-protein, appears the most promising with high capacity to interact with the chondrocytes. Another protein with ability to interact with the chondrocyte is the 58kDa-protein. This protein is common to all connective tissues. We have no information on the function of these proteins in the matrix. One interesting possibility is modulation of chondrocyte behaviour such as altered synthesis of matrix components, a question which is presently being investigated.

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Finally, a comment by our son Fredrik (5 months) in his own handwriting with the aid of the portable computer on which this thesis have been written: "bnm hmhjbuvuyn v ygygvvgvcv rSA B BVYCV "

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Specific interaction between cartilage proteoglycans and hyaluronic acid at the chondrocyte cell surface

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Binding of exogenous [35 S]sulphate-labelled cartilage proteoglycans to cells was studied with suspension cultures of calf articular-cartilage chondrocytes. Proteoglycans interact with hyaluronic acid at the cell surface via their hyaluronic acid-binding region. The binding is time-dependent and saturable, but does not appear to be freely reversible. The bound 35 S-labelled proteoglycans are located at the cell surface, and only small proportions of the proteoglycans are internalized.

Chondrocytes cultured *in vitro* retain their capacity to synthesize and secrete proteoglycans. *In vivo* as well as *in vitro* most of the proteoglycans participate in the extracellular formation of large aggregates together with hyaluronic acid and link protein (Hascall & Heinegård, 1974*a,b*; Kimura *et al.*, 1979, 1980; Björnsson & Heinegård, 1981*b*). A substantial proportion of the proteoglycans is, however, associated with the cell surface, even in single-cell suspension cultures (Björnsson & Heinegård, 1981*a*). The relative size of this cell-associated pool varies with the culture system used, and the mechanism for the binding of proteoglycans to the chondrocyte cell surface is not known. The turnover of these proteoglycans is slow (Mikuni-Takagaki & Toole, 1979; Björnsson & Heinegård, 1981*a*), making it unlikely that the pool of proteoglycans at the cell surface only represents an intermediate step in the export into the medium.

Binding of proteoglycans to cells has been studied with other cell types. In the case of heparan sulphate proteoglycans bound to the surface of hepatocytes, the interaction is mediated by two different mechanisms. One fraction binds via the polysaccharide side chains to a cell-surface receptor, and another is bound via a hydrophobic region in the proteoglycan core protein, which is believed to be integral in the membrane (Kjellén *et al.*, 1980, 1981). Other studies have shown that fibroblast proteoglycans can bind to fibroblasts, probably via their protein core (Prinz *et al.*, 1978). These proteoglycans were internalized, however, and therefore represent a degradation pool rather than a structural one.

In the present investigation we have studied the ability of chondrocytes in suspension culture to bind exogenous cartilage proteoglycans. Such binding of proteoglycans to the chondrocyte surface could be

an important part of a feedback control on chondrocyte cell function exerted by the extracellular matrix.

Experimental

Materials

The medium used for spinner culture was Ham's F12 medium supplemented with 50× essential amino acids (for Eagle's minimum essential medium; Flow Laboratories, Rockville Pike, MD, U.S.A.) (20 ml/l), dextran sulphate (20 µg/ml) (Pharmacia Fine Chemicals, Uppsala, Sweden), penicillin (Flow Laboratories) (50 i.u./ml) and streptomycin (Flow Laboratories) (50 i.u./ml) and buffered with 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.), pH 7.4. For binding experiments Ham's F12 medium buffered with 20 mM-Hepes at pH 7.4 was used. Collagenase CLS was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), and *Streptomyces hyaluronidase* was from Miles Laboratories (Elkhart, IN, U.S.A.). Conical polypropylene tubes (50 ml) and bacteriological Petri dishes were from Nunc (Roskilde, Denmark). For binding experiments 2 ml polypropylene vials (no. 72.708; Sarstedt, Numbrecht, W. Germany) for the Eppendorf 3200 centrifuge were used.

Articular-cartilage proteoglycans were prepared from metacarpophalangeal joints of 6-month-old calves. These proteoglycans were extracted with 4 M-guanidinium chloride/5 mM-N-ethylmaleimide/50 mM sodium acetate buffer, pH 5.8, containing the protein inhibitors 5 mM-benzamidine chloride, 0.1 M-6-aminohexanoic acid and 10 mM-EDTA. Solid CsCl was added to the extract to increase the density to 1.50 g/ml. The proteoglycans were isolated by equilibrium centrifugation in an MSE

6 × 14 ml swinging-bucket rotor at 30000 rev./min (110000 g_{av}) for 65 h at 18°C. The bottom quarter of the tube was separated with a Beckman tube slicer. This fraction had a density of 1.61 g/ml and contained the bulk of the proteoglycans. Nasal septum-cartilage proteoglycan aggregates, hyaluronic acid-binding region and a mixture of chondroitin sulphate-attachment region and keratan sulphate-attachment region were prepared as described previously (Heinegård & Axelsson, 1977). Human umbilical-cord hyaluronic acid was from Sigma Chemical Co. Hyaluronic acid oligomers were prepared by the method of Hascall & Heinegård (1974b).

Isolation of cells

Articular-cartilage chondrocytes were prepared from metacarpophalangeal joints from forelegs of 6-month-old calves. Hyaline cartilage was dissected from all articular-cartilage surfaces of the joint and cut into small pieces. The cell-suspension procedure was essentially the one described by Björnsson & Heinegård (1981a) for the preparation of foetal-calf tracheal chondrocytes. In short, the chondrocytes were liberated by collagenase digestion (0.2%, w/v) under constant agitation for 9 h at 37°C. The cells were washed and transferred to spinner bottles containing Ham's F12 medium with supplements. The cells were kept in spinner culture at 37°C for 2 days to allow them to reconstitute their cell surface.

Preparation of [³⁵S]sulphate-labelled proteoglycans

Chondrocytes (0.5×10^6 cells/ml) were cultured in Ham's F12 medium containing 20 mM-Hepes, pH 7.5, and 50 i.u. of penicillin/ml on hydrophobic bacteriological Petri dishes. Carrier-free [³⁵S]sulphate (The Radiochemical Centre, Amersham, Bucks., U.K.) (20 μ Ci/ml) was added. After 6 days the medium was collected and concentrated by ultrafiltration (PM10; Amicon, Lexington, MA, U.S.A.). An equal volume of 8 M-guanidinium chloride was added to the concentrated medium, followed by solid CsCl to give a density of 1.50 g/ml. The [³⁵S]-labelled proteoglycans were recovered from the bottom quarter of the tubes after equilibrium centrifugation, as described above. Proteoglycan preparations were dialysed against water, freeze-dried and dissolved in medium to make a concentrated stock solution for use in binding experiments.

Binding experiments

Cells were collected from spinner culture by centrifugation, washed twice with Ham's F12 medium, and suspended in fresh medium (containing no dextran sulphate) at a concentration of 2×10^6 cells/ml essentially as described previously

(Björnsson & Heinegård, 1981b). Samples (1 ml) of the cell suspension were pipetted into 2 ml polypropylene vials that had been precoated with bovine serum albumin (1 mg/ml in distilled water for 15 h at 20°C) to minimize non-specific binding to the walls. The cells were incubated for 1.5–2 h on a gyrating board (Nutator; Becton and Dickinson Labware, Oxnard, CA, U.S.A.) before the start of the experiment.

Addition of labelled proteoglycans was done either by pipetting 10 μ l of a concentrated stock solution directly into the cell suspension or by suspending cells that had been pelleted (30 s in an Eppendorf model 3200 centrifuge operated at 90 V) in 1 ml of fresh medium containing labelled proteoglycans. The amounts of proteoglycan bound were similar in the different procedures. When desired, competitors for binding were also added to the medium containing labelled proteoglycans. After incubation at 37°C on the gyrating board, the cells were pelleted by centrifugation and the medium was removed for determination of radioactivity. The cells were washed once by resuspending them in fresh medium followed by centrifugation, and this rinse was discarded. The efficiency of the rinsing procedure would allow less than 0.05% of non-bound proteoglycans to be recovered with the cell pellet. This pellet was finally suspended in 1 ml of Dulbecco's phosphate-buffered saline, pH 7.3 (Flow Laboratories), and samples were taken for determination of radioactivity. Before liquid-scintillation counting, samples of media and cells were made 1% (w/v) with respect to sodium dodecyl sulphate, heated at 60°C for 30 min and mixed with scintillation 'cocktail' (Instagel; Packard, Downers Grove, IL, U.S.A.) in a ratio of 1:10.

Results and discussion

Characterization of [³⁵S]sulphate-labelled proteoglycans

Preparations of [³⁵S]-labelled proteoglycans were characterized by gel chromatography on Sepharose 2B. The major proportion of the proteoglycans was eluted as high- M_r monomers (Heinegård, 1972) (Fig. 1a). The ability of these labelled monomers to form aggregates with hyaluronic acid was demonstrated by the shift of a large proportion to the void volume after incubation with hyaluronic acid (2%, w/w) (Fig. 1b). The presence of the keratan sulphate-attachment region, typical for cartilage proteoglycans (Heinegård & Axelsson, 1977), was demonstrated by gel chromatography on Sepharose 6B of labelled proteoglycans sequentially digested with chondroitinase ABC and trypsin (results not shown). The [³⁵S]-labelled proteoglycan preparations used in this study thus contained typical cartilage proteoglycan monomers capable of forming aggregates

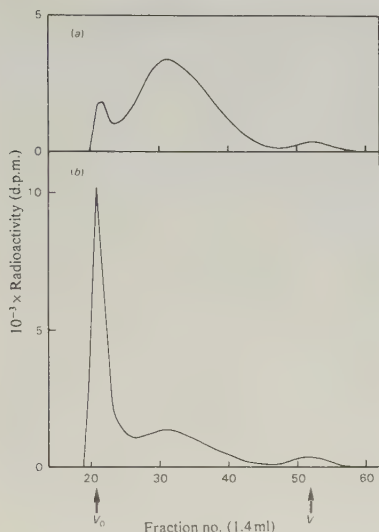


Fig. 1. Sepharose 2B chromatography of ^{35}S -sulphate labelled proteoglycans

(a) The isolated ^{35}S -labelled proteoglycans and carrier bovine tracheal-cartilage proteoglycan monomer were dissolved in 0.5 M sodium acetate buffer, pH 7.0. The sample was chromatographed on a column (140 cm $\times$ 0.8 cm) of Sepharose 2B eluted with 0.5 M sodium acetate buffer, pH 7.0. (b) Hyaluronic acid was added [2% (w/w) of proteoglycan] to an identical sample and left at room temperature for 24 h before chromatography as above. V_0 , Void volume; V_t , total volume.

with hyaluronic acid. With time in storage the properties of the ^{35}S -labelled proteoglycans changed, as demonstrated by impaired aggregation with hyaluronic acid as well as decreased cell binding. This could possibly be due to radiolysis.

Kinetics of binding of ^{35}S -sulphate-labelled proteoglycans to cells

In initial experiments it was shown that when the ^{35}S -labelled proteoglycans were incubated with chondrocytes in suspension culture about 5% of the added molecules were bound and could be recovered with the cells (Fig. 2). The amount of ^{35}S -labelled proteoglycans that was bound to the chondrocytes increased rapidly during the first 2 h of incubation. The rate at which additional proteoglycans bound then decreased, and maximum binding was slowly reached within 5 h.

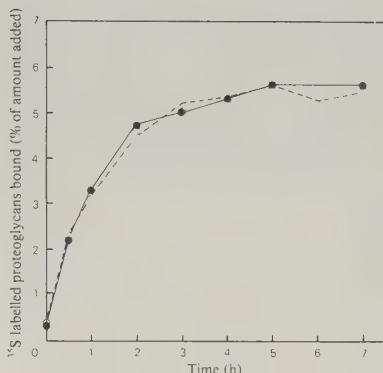


Fig. 2. Time course of binding of ^{35}S -sulphate-labelled proteoglycans

Cells were pelleted by centrifugation and resuspended in 1 ml of medium containing ^{35}S -labelled proteoglycans (O, 1 $\mu\text{g/ml}$; ●, 5 $\mu\text{g/ml}$). The amount of ^{35}S -labelled proteoglycans associated with cells was determined after incubation as described in the Experimental section.

Localization of bound ^{35}S -sulphate-labelled proteoglycans

To determine to what extent the bound proteoglycans were cell-surface-associated, the cells were allowed to bind ^{35}S -labelled proteoglycans for 2–5 h and were then treated briefly with trypsin (for details see Fig. 3 legend). At all times more than 86% of bound ^{35}S label was released by the digestion (Fig. 3), indicating a cell-surface localization of bound ^{35}S -labelled proteoglycans. Only a minor proportion was resistant to trypsin digestion and was recovered with the cell fraction, possibly representing internalized proteoglycans. This supports earlier studies on endocytosis of proteoglycans by chicken chondrocytes (von Figura *et al.*, 1980). These cells bound cartilage proteoglycans, which, however, were not internalized.

Concentration-dependence of binding of ^{35}S -sulphate-labelled proteoglycans

To investigate if the capacity of the chondrocytes to bind proteoglycans is saturable, various amounts of ^{35}S -labelled proteoglycans were added to 2×10^6 cells in 1 ml of medium and incubated for 2 h. There was no sharp transition in amount bound when increasing amounts of ^{35}S -labelled proteoglycans were added (Fig. 4a). If the relative proportion of added proteoglycans bound to cells is considered, rather than the absolute amount, there was a marked

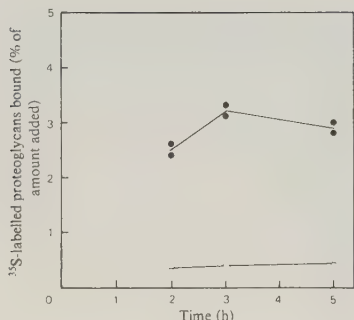


Fig. 3. Localization of bound [³⁵S]sulphate-labelled proteoglycans

³⁵S-labelled proteoglycans were allowed to bind to chondrocytes for the times indicated. The cells were then washed with fresh medium as described in the Experimental section, and resuspended in Ham's F12 medium containing 0.1% trypsin. Digestions were performed for 15 min at 3°C on a Nutator to prevent aggregation of the cells. Cells (trypsin-resistant material, ○) and medium (trypsin-releasable material, ●) were separated by centrifugation. The cells were then suspended in Dulbecco's phosphate-buffered saline, and samples of cell suspension and medium were taken for determination of radioactivity. Viability was better than 95% after trypsin treatment, as judged by Trypan Blue exclusion.

decrease in the proportion bound at concentrations above 16 µg/ml (Fig. 4b). It appears that the saturation of the binding is not absolute, but that, when a certain number of proteoglycans are bound, additional molecules bind with less affinity, and possibly by other mechanisms. If the binding of proteoglycans to the cell surface is part of the construction of an extracellular matrix, no sharp division between cell-surface proteoglycans and extracellular matrix should be expected. In agreement, electron microscopy of articular-cartilage cells in monolayer culture shows a diffuse layer containing proteoglycans and collagen at the cell surface of the chondrocytes, indicating establishment of an extracellular matrix (Kuettnner *et al.*, 1982).

Specificity of binding of [³⁵S]sulphate-labelled proteoglycans

Unlabelled proteoglycans, isolated proteoglycan substructures and hyaluronic acid were used as competitors for the binding of ³⁵S-labelled proteoglycans to chondrocytes (Fig. 5). Both articular-cartilage and nasal-septum-cartilage (results not shown) proteoglycan monomers efficiently inhibited binding of the ³⁵S-labelled proteoglycans (Fig. 5a). About 50 µg of articular-cartilage monomer is needed to give 50% inhibition of binding when 4 µg of ³⁵S-labelled proteoglycans is present. One possible explanation for this is that these two preparations contain different proportions of two or more types of proteoglycans that differ in their cell-binding properties. Nasal-septum-cartilage proteoglycan aggregate

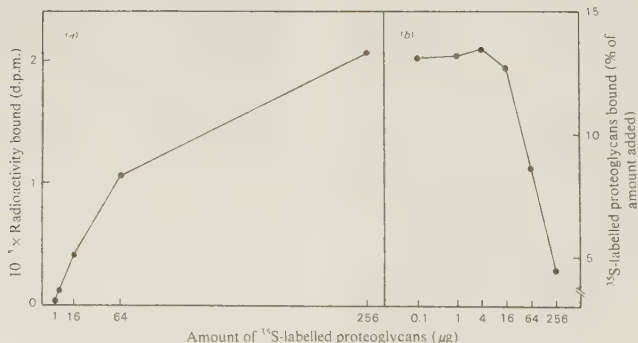


Fig. 4. Saturation of binding of [³⁵S]sulphate-labelled proteoglycans to chondrocytes

³⁵S-labelled proteoglycans were added by resuspending pelleted cells in medium containing ³⁵S-labelled proteoglycan at the concentrations indicated. After 2 h the incubation was terminated as described in the Experimental section. (a) Absolute values for bound ³⁵S-labelled proteoglycan. (b) Proportion of ³⁵S-labelled proteoglycan bound expressed as percentage of the amount added.

preparation (A1) containing mainly link-protein-stabilized aggregates was a less efficient competitor (Fig. 5b). Compared with proteoglycan monomers, about 7 times more (by wt.) of the proteoglycan aggregate preparation was needed to achieve 50% inhibition of binding of ^{35}S -labelled proteoglycans. Nasal-septum-cartilage A1 preparations have been shown to contain about 15% proteoglycan monomers (Heinegård & Hascall, 1979), since a substantial portion of these monomers can interact with hyaluronic acid *in vitro*, it appears that the inhibition obtained with this preparation could be due solely to the presence of free monomer.

Addition of reduced and alkylated articular-cartilage proteoglycan monomer ($64\text{ }\mu\text{g/ml}$) decreased the binding of ^{35}S -labelled proteoglycans to 75% of the control value, whereas intact monomers at this concentration decreased binding to 40% (Fig. 5a). Because reduction of monomers will only modify the hyaluronic acid-binding region (Heinegård, 1977), it appeared that this structure may be involved in the binding to cells.

Various substructures of proteoglycan monomer were used in the competition assay to identify the part of the molecule that binds to the cell surface.

Isolated hyaluronic acid-binding region competed efficiently with the ^{35}S -labelled proteoglycans for binding (Fig. 5c). On the other hand, a preparation containing a mixture of chondroitin sulphate-attachment region and keratan sulphate-attachment region (A1:T:A1; Heinegård & Axelsson, 1977) did not compete for binding (Fig. 5d). A somewhat increased binding of ^{35}S -labelled proteoglycans in the presence of chondroitin sulphate-attachment region and keratan sulphate-attachment region seems to be a general effect of anionic polymers, since dextran sulphate gave a similar, but even more pronounced, effect (Fig. 5g).

The hyaluronic acid-binding region was the only efficient competitor among the proteoglycan substructures, suggesting that the ^{35}S -labelled proteoglycans bind to the cell by this part of the molecule. Furthermore, when ^{35}S -labelled proteoglycans were reduced and alkylated to unfold the hyaluronic acid-binding region, binding was decreased to 2% compared with that of untreated proteoglycans (results not shown). Assuming, then, that proteoglycans bind to chondrocytes by their hyaluronic acid-binding region, a probable receptor structure is hyaluronic acid at the cell surface. If so, hyaluronic

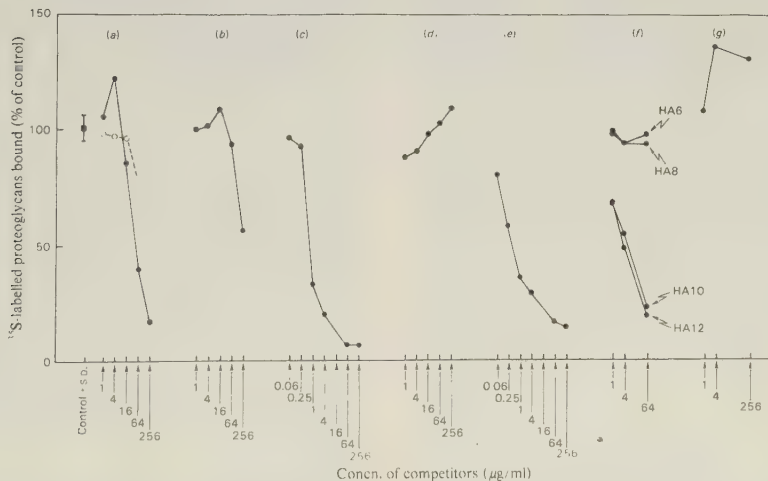


Fig. 5. Competition for binding of [^{35}S]sulphate labelled proteoglycans

Additions of $4\text{ }\mu\text{g}$ of ^{35}S labelled proteoglycan and competitors were made simultaneously by change of medium as described in the Experimental section. Incubation was for 3 h. ■, Control without competitors added. Bars indicate $\pm$ s.d. ($n=3$). (a) ●, Articular-cartilage aggregate; ○, reduced and alkylated articular cartilage monomer preparation; (b) nasal-septum-cartilage aggregate; (c) hyaluronic acid-binding region; (d) chondroitin sulphate attachment region and keratan sulphate-attachment region; (e) hyaluronic acid oligomers containing indicated number of monosaccharide residues; (f) hyaluronic acid; (g) dextran sulphate.

acid should be an efficient competitor, as was indeed the case (Fig. 5e). Further support for this mechanism for attachment was obtained by determining the size of hyaluronic acid required for efficient competition. The decasaccharide was the smallest hyaluronic acid oligosaccharide to compete efficiently (Fig. 5f), exactly as has been previously described for binding of proteoglycan monomer to hyaluronic acid in the formation of cartilage proteoglycan aggregates (Hardingham & Muir, 1973; Hascall & Heinegård, 1974b).

Release of cell-bound [35 S]sulphate-labelled proteoglycans by treatment with Streptomyces hyaluronidase

The role of hyaluronic acid at the cell surface in the binding of 35 S-labelled proteoglycans was further studied by specific enzymic degradation. Digestion of chondrocytes with *Streptomyces* hyaluronidase released more than 95% of 35 S-labelled proteoglycans that had been bound to the cells (Table 1). To inhibit the proteolytic activity known to be present in the hyaluronidase preparation (Caputo *et al.*, 1980), ovomucoid plus newborn-calf serum, known to contain several proteinase inhibitors, was included in digestion buffers. Under such conditions the 35 S-labelled proteoglycan was not degraded during digestion, as judged by Sepharose 2B chromatography (results not shown). Furthermore, the aggregating capacity of the 35 S-labelled proteoglycans remained. No proteolytic activity was

detected in the mixture of hyaluronidase and proteinase inhibitors, with [3 H]acetyl-labelled haemoglobin as the substrate. The detection limit of this assay is low, corresponding to 0.1 ng of trypsin/ml. A 100 fold higher amount of trypsin (10 ng) than the highest possible proteinase activity of the digestion mixture containing 1 turbidity-reducing unit of hyaluronidase did not release any 35 S label from the cells (Table 1). The almost complete removal of bound 35 S-labelled proteoglycans by digestion with *Streptomyces* hyaluronidase demonstrates that the 35 S-labelled proteoglycans are indeed bound to hyaluronic acid at the cell surface.

Reversibility of binding of [35 S]sulphate-labelled proteoglycans to chondrocytes

The binding of proteoglycans to hyaluronic acid in proteoglycan aggregates is stabilized by link protein (Heinegård & Hascall, 1974; Kimura *et al.*, 1979; Hardingham, 1979). Such link-protein-stabilized aggregates are stable to competition with hyaluronic acid or proteoglycan monomers. Proteoglycans at the cell surface of chondrocytes have a slow turnover (Mikuni-Takagaki & Toole, 1979; Björnsson & Heinegård, 1981b). It is therefore possible that proteoglycan binding to hyaluronic acid at the chondrocyte cell surface is stabilized, possibly by link proteins. If so, they should not be readily displaced by either exogenous or newly synthesized proteoglycans. To investigate this possibility, 35 S-labelled proteoglycans were allowed to bind to the cells for 1 h and 4 h. Attempts were then made to displace bound proteoglycans by the addition of fresh medium containing the same competitors as used before (Fig. 6). In control cultures, bound 35 S-labelled proteoglycans decreased to 75% of the initial value during 3 h, i.e. 25% of bound proteoglycans were released. Competitors added to the cells at a concentration that would effectively compete for the initial binding resulted in release of only 39% of the 35 S-labelled proteoglycans. There was no significant difference between the results when 35 S-labelled proteoglycans were allowed to bind for 1 h or 4 h, other than the total amount bound. Thus binding of 35 S-labelled proteoglycans is not a freely reversible process. It is likely that the binding is stabilized by an additional factor, possibly link proteins.

General discussion

The present study has shown that cartilage proteoglycans bind via their hyaluronic acid-binding region to hyaluronic acid at the chondrocyte cell surface and that the binding is stable towards competition. It is possible that this hyaluronic acid-proteoglycan complex is stabilized by link proteins, in analogy to the proteoglycan aggregates

Table 1. Release of cell-bound [35 S]sulphate-labelled proteoglycan by *Streptomyces* hyaluronidase

Labelled proteoglycan (4 μ g) was allowed to bind to chondrocytes in 1 ml of medium for 3 h. Enzymes and/or proteinase inhibitors [20 μ g of ovomucoid and 10% (v/v) newborn-calf serum] were then added in 125 μ l of medium, and the samples were incubated for an additional 1 h before the determination of the amount of cell-attached 35 S-labelled proteoglycan. The values given are the means of two determinations. Abbreviation: TRU, turbidity-reducing unit (Ohya & Kaneko, 1970).

Addition	35 S radioactivity bound to cells (d.p.m.)
Medium	27 150
Medium with proteinase inhibitors	24 357
Medium with 1 TRU of <i>Streptomyces</i> hyaluronidase + proteinase inhibitors	1060
Medium with 0.1 TRU of <i>Streptomyces</i> hyaluronidase + proteinase inhibitors	1492
Medium with 0.01 TRU of <i>Streptomyces</i> hyaluronidase + proteinase inhibitors	2139
Medium with 100 ng of trypsin	4963
Medium with 10 ng of trypsin	26 009
Medium with 1 ng of trypsin	24 770

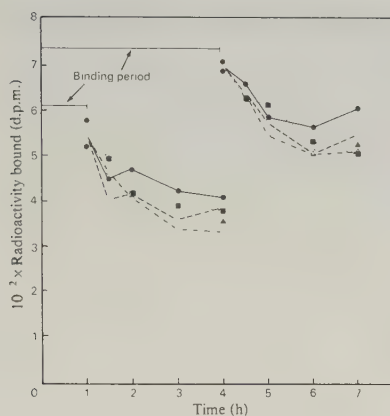


Fig. 6. Reversibility of binding of [^{35}S]sulphate-labelled proteoglycans to chondrocytes

^{35}S -labelled proteoglycans ($5\mu\text{g}$ in $10\mu\text{l}$ of stock solution) were added to the cell suspension. After 1 h or 4 h of incubation the cultures were centrifuged. The medium was withdrawn and the cells were resuspended in fresh medium with or without competitors. Concentrations of competitors were chosen to give about 80% inhibition if added with the labelled proteoglycan (cf. Fig. 5). Incubations were terminated as described in the Experimental section. ●, Control without competitors; □, articular cartilage monomer ($256\mu\text{g}$); ■, hyaluronic acid-binding region ($4\mu\text{g}$); △, hyaluronic acid dodecasaccharide ($64\mu\text{g}$); ▲, hyaluronic acid ($16\mu\text{g}$).

formed in the medium of chondrocyte cultures and present in cartilage matrix. Internalization is not a major process. Therefore, during the time period studied, the added ^{35}S -labelled proteoglycans seem to be integrated as a part of the pericellular matrix. The proteoglycan molecules in this matrix appeared to be bound to the cells by specific bonds, since reduced and alkylated ^{35}S -labelled proteoglycans showed no binding.

The different binding of proteoglycans isolated from cultured chondrocytes and isolated from cartilage is puzzling in view of the fact that both preparations have equal capacity to bind to hyaluronic acid *in vitro*. It may be that the preparations contain different proportions of the two subpopulations of aggregating proteoglycans (Heinegård *et al.*, 1981), or that proteoglycans are modified with time in the extracellular matrix, such that their hyaluronic acid-binding regions bind less strongly, either to hyaluronic acid or to stabilizing factors.

In cartilage chondrocytes are embedded in an

extensive extracellular matrix, containing predominantly collagen and proteoglycans. The chondrocytes seem to be able to regulate the content of macromolecules in this matrix (Hardingham *et al.*, 1972), by an unknown mechanism. It is possible that feedback control by an interaction between proteoglycans in the matrix and a cell-surface receptor for proteoglycans play a role (Nevo & Dorfman, 1972; Wiebkin & Muir, 1977; Lowther & Handley, 1979). Other potential regulating factors are hormonal influences, mechanical properties of the matrix, and interactions with collagen and other matrix components. Suspension cultures are well suited for identification of specific cell-surface receptors for proteoglycans, as the cells do not accumulate an extracellular matrix as in monolayer cultures. The approach used does not exclude additional mechanisms for proteoglycan association with the chondrocyte. There may be a pool of proteoglycans bound to the cell surface by a different mechanism.

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Four classes of cell-associated proteoglycans in suspension cultures of articular-cartilage chondrocytes

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The characteristics of cell-associated proteoglycans were studied and compared with those from the medium in suspension cultures of calf articular-cartilage chondrocytes. By including hyaluronic acid or proteoglycan in the medium during [35 S]sulphate labelling the proportion of cell-surface-associated proteoglycans could be decreased from 34% to about 15% of all incorporated label. A pulse chase experiment indicated that this decrease was probably due to blocking of the reassociation with the cells of proteoglycans exported to the medium. Three peaks of [35 S]sulphate-labelled proteoglycans from cell extracts and two from the medium were isolated by gel chromatography on Sephacryl S-500. These were characterized by agarose/polyacrylamide-gel electrophoresis, by SDS/polyacrylamide-gel electrophoresis of core proteins, by glycosaminoglycan composition and chain size as well as by distribution of glycosaminoglycans in proteolytic fragments. The results showed that associated with the cells were (a) large proteoglycans, typical for cartilage, apparently bound to hyaluronic acid at the cell surface, (b) an intermediate-size proteoglycan with chondroitin sulphate side chains (this proteoglycan, which had a large core protein, was only found associated with the cells and is apparently not related to the large proteoglycans), (c) a small proteoglycan with dermatan sulphate side chains with a low degree of epimerization, and (d) a somewhat smaller proteoglycan containing heparan sulphate side chains. The medium contained a large aggregating proteoglycan of similar nature to the large cell-associated proteoglycan and small proteoglycans with dermatan sulphate side chains with a higher degree of epimerization than those of the cells, i.e. containing some 20% iduronic acid.

INTRODUCTION

Chondrocytes in cartilage are surrounded by a predominant extracellular matrix mainly composed of collagen and proteoglycan. Little is known about the interactions of the matrix components with the cell surface, although they are probably important for the regulation of matrix turnover. A receptor for type II collagen has been identified (Mollenhauer & von der Mark, 1983), and proteoglycans can specifically bind to hyaluronic acid at the cell surface (Sommarin & Heinegård, 1983). Furthermore the biosynthetic activity of chondrocytes in culture can be modulated by proteoglycans or hyaluronic acid added to the culture medium (Nevo & Dorfman, 1972; Schwartz & Dorfman, 1975; Handley & Lowther, 1977; Wiebkin & Muir, 1977; Solursh *et al.*, 1980). In studies performed *in vivo* with young rabbits (Thomas, 1956) or in organ culture with chick-embryo cartilage sections (Hardingham *et al.*, 1972), chondrocytes were able to regulate the amount of proteoglycan in the extracellular matrix and replace any such lost upon enzyme treatment. This regulatory function may be mediated via interactions at the cell surface. The structural basis for such a mechanism is, however, not known, in part owing to the fact that the macromolecules present at the chondrocyte cell surface have not been characterized.

In a previous study we used suspension cultures of calf articular-cartilage chondrocytes to study the interaction of added proteoglycans with hyaluronic acid at the chondrocyte cell surface (Sommarin & Heinegård, 1983). In the present study a similar interaction of endogenously

synthesized proteoglycans was studied and proteoglycans bound by other mechanisms at the cell surface were isolated and characterized.

EXPERIMENTAL

Materials

The medium used for spinner culture was Ham's F12 medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 20 μ l of 50 $\times$ essential amino acids (for Eagle's minimum essential medium; Flow Laboratories, Bethesda, MD, U.S.A.)/ml, 20 μ g of dextran sulphate (Pharmacia Fine Chemicals, Uppsala, Sweden)/ml, 50 i.u. each of penicillin and streptomycin (Flow Laboratories)/ml and buffered with 20 mM-Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.), pH 7.4.

Radiochemicals were obtained from Amersham International (Amersham, Bucks, U.K.). Other chemicals were of analytical-reagent grade and obtained from standard commercial sources.

Cell culture

Chondrocytes were prepared from calf articular cartilage from the metacarpophalangeal joint as described previously (Sommarin & Heinegård, 1983). In short, cartilage was digested with collagenase and the liberated cells were washed and transferred to spinner culture in Ham's F12 medium with supplements. The cells were kept in spinner culture to allow reconstitution of their cell surface.

At the time of the experiment, i.e. after 36 h in spinner culture, the cells were transferred to Ham's F12 medium

Abbreviation used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate.

buffered with Hepes but without other supplements. Radiolabelling was performed at 37 °C in 2 ml polypropylene test tubes (Sarstedt, Numbrecht, W. Germany) with 1 ml of medium containing 5×10^6 cells. The cells were kept in suspension with gentle agitation on a gyrating board (Nutator; Becton and Dickinson Labware, Oxnard, CA, U.S.A.). After labelling, the cells were pelleted by centrifugation in an Eppendorf 3200 centrifuge as described elsewhere (Björnsson & Heinegård, 1981), and the medium was removed. The cells were washed once by suspension in ice-cold medium and pelleted again, the wash medium was discarded and the cells were directly treated with trypsin or kept on ice for no more than 20 min before extraction.

Analysis of competition experiments

In the first two experiments (presented in Table 1 and Fig. 1) the cells were digested with trypsin (0.1%, w/v). To minimize biosynthetic activity a short time (15 min) and a low temperature (4 °C) were used. The cells were then pelleted, and the solubilized material was removed. The cell pellet was dissolved in 1 ml of 0.5 M-NaOH at 65 °C for 6 h. This solution was neutralized by the addition of 10 M-acetic acid. Cell viability after trypsin treatment was above 95% as judged by the Trypan Blue-exclusion test.

Proteoglycans were separated from free radiolabelling compound by cetylpyridinium chloride precipitation. Samples with added carrier chondroitin 6-sulphate (100 µg) were precipitated with 0.5% cetylpyridinium chloride, 0.6 M-NaCl, 20 mM- Na_2SO_4 and 50 mM-sodium acetate overnight at room temperature. The samples were centrifuged and the pellet was washed once with the above buffer. The pellet was solubilized with 4 M-guanidinium chloride, and Instagel (Packard, La Grange, IL, U.S.A.) was added for scintillation counting of radioactivity.

Extraction of cells

In other experiments trypsin digestion of cells was omitted and instead cell-bound material was extracted by a procedure involving the use of detergent. First 500 µl of 8% CHAPS/0.1 M-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors EDTA (20 mM), 6-aminohexanoic acid (0.2 M), benzamidine chloride (10 mM), *N*-ethylmaleimide (10 mM) and phenylmethanesulphonyl fluoride (2 mM) was added to the cell pellet and the sample was vigorously stirred. After 15 min at 4 °C an equal volume of 8 M-guanidinium chloride was added and extraction was continued for 45 min. The non-extractable material was removed by centrifugation at 105000 $g_{av.}$ for 1 h at 4 °C in a 70.1 Ti angle rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). The extraction yield with this procedure was about 85% of all [^{35}S]sulphate-labelled material. Increasing the extraction time to 24 h did not increase the extraction yield. The proteolytic activity in these extracts was negligible, since a 24 h incubation of the extracts at room temperature did not change the elution profile of labelled components on Sephacryl S-500.

Analytical Sephacryl S-500 gel chromatography

Cell extracts or medium were chromatographed on a Sephacryl S-500 column (140 cm $\times$ 0.6 cm) eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-

sodium acetate buffer, pH 5.8. Fractions (0.7 ml) were collected and assayed for radioactivity.

Preparation of proteoglycan populations

Cells were incubated for 4 h in Ham's F12 medium with 1 mg of hyaluronic acid (human umbilical cord; Sigma Chemical Co.)/ml and 50 µCi of [^{35}S]sulphate/ml. The sulphate concentration was then adjusted to 3 mM, i.e. a 500-fold excess of non-radioactive sulphate was added from a 0.15 M stock solution. The cells were then chased in this medium for 20 min, pelleted and rinsed with fresh medium. They were then extracted with CHAPS and guanidinium chloride as described above. Both extract and medium were centrifuged as above to remove insoluble material and were then kept frozen at -60 °C until analysis.

Samples of extracts were transferred to 6 M-urea/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors listed above by gel filtration on Sephadex G-50 columns (10 ml) eluted with this solution. Samples of medium were adjusted to 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8, before being applied to the column. The void-volume fraction containing the proteoglycans was directly applied to a small ion-exchange column (3 cm $\times$ 0.8 cm) of DEAE-cellulose (Whatman DE52) in 6 M-urea/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. The column was eluted with 2×3 ml of 6 M-urea/0.5% Triton X-100/0.32 M-sodium acetate buffer, pH 5.8, to remove most of the protein in the samples. Proteoglycans were eluted with 3 ml of 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8.

Purified proteoglycans were fractionated by chromatography on a preparative Sephacryl S-500 column (1.6 cm $\times$ 142 cm) eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. Fractions (4 ml) were collected and assayed for radioactivity. The chromatogram was pooled as indicated in Figs. 3(a) and 3(b).

Agarose/polyacrylamide-gel electrophoresis

Fractions were pooled from a Sephacryl S-500 chromatogram of an extract, where the ion-exchange chromatography was omitted. Samples were precipitated with 5 vol. of ethanol overnight at 4 °C, with bovine articular-cartilage proteoglycan monomer (20 µg of an A1 preparation) as carrier. The precipitates were dissolved in 0.5% SDS/10% (w/v) sucrose/1 mM Na_2SO_4 /10 mM-Tris/acetate buffer, pH 6.8, for 4 h at 37 °C and electrophoresed on a slab gel of agarose/polyacrylamide essentially as described by Heinegård *et al.* (1985b). After fixing and staining of the gel with Toluidine Blue the radioactive bands were detected by fluorography (Chamberlain, 1979).

Glycosaminoglycan characterization

Proteoglycans in samples of pooled fractions were precipitated with 5 vol. of ethanol, with 100 µg of chondroitin 6-sulphate as a carrier. After centrifugation, the pellet was dissolved in 0.5 M-NaOH and incubated at 4 °C for 16 h. After neutralization with 10 M-acetic acid, the liberated glycosaminoglycan chains were precipitated with 9 vol. of 95% ethanol overnight at 4 °C. The precipitates were dissolved in 0.1 M-Tris/0.1 M-sodium acetate buffer, pH 7.6. The recovery in this procedure was

better than 90%. Samples were digested with 0.02 nominal unit ($\mu\text{mol}/\text{min}$) of either chondroitinase ACII or chondroitinase ABC at 37 °C for 5 h or subjected to HNO_2 treatment by the method of Shively & Conrad (1976). Treated and untreated control samples were then chromatographed on a Sephadex G-50 column (1 cm $\times$ 6.5 cm) eluted with 4 M-guanidinium chloride/50 mM-sodium acetate, buffer, pH 5.8, to separate disaccharides or short oligosaccharides (close to total volume) formed during enzyme or HNO_2 treatment from large resistant fragment (void volume). Fractions were collected directly into scintillation counting vials and radioactivity was determined.

Size of glycosaminoglycan side chains

Samples were precipitated with ethanol as for glycosaminoglycan analysis and treated with alkaline borohydride as described by Carlsson (1968). The size distribution of the glycosaminoglycan chains was then determined by gel chromatography on a Superose 6B column (0.65 cm $\times$ 92 cm) eluted with 0.2 M- Na_2SO_4 . Fractions (480 μl) were collected and assayed for radioactivity. The column was calibrated with chondroitin sulphate standards of known M_r values, which allowed the calculation of weight-average and number-average M_r values in accordance with Wasteson (1971).

Size of fragments produced by trypsin digestion

Samples were precipitated with ethanol as above but with 0.5 mg of bovine nasal-septum-cartilage proteoglycan monomer as carrier. The precipitates were solubilized in 0.1 M-Tris/acetate buffer, pH 7.6, and treated with 5 μg of trypsin [L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated; Sigma Chemical Co.] for 5 h at 37 °C. The digests were chromatographed on the Superose 6B column used for chain-size determination but were eluted with 0.5 M-sodium acetate buffer, pH 7. Carrier proteoglycan uronic acid was determined by the carbazole reaction, with an automated system (Heinegård, 1973).

Characterization of core proteins by SDS/polyacrylamide-gel electrophoresis

Cells were incubated with 50 μCi of [^3H]leucine and 10 μCi of [^{35}S]sulphate/ml in Ham's F 12 medium containing 1 mg of hyaluronic acid/ml. Proteoglycan populations were prepared essentially as before, but additional purification had been obtained by a second ion-exchange chromatography on DEAE-cellulose eluted with a 0.05–1 M-NaCl linear gradient in 7 M-urea/0.5% Triton/50 mM-sodium acetate buffer, pH 4 (results not shown). Samples were precipitated with 5 vol. of ethanol, and pellets were dissolved in 50 M-Tris/HCl buffer, pH 7.6, and digested with 0.02 unit of chondroitinase ABC and/or 0.02 unit of heparitinase with 5 μg of ovomucoid for 1 h at 37 °C. The samples were then electrophoresed on SDS/polyacrylamide gradient gels (3–10%) with the buffer system of Laemmli (1970). Radioactivity was detected by fluorography as above.

RESULTS

Prevention of binding of newly synthesized proteoglycan to cells by competition with hyaluronic acid or proteoglycan monomer

In control cultures as much as 34% of the newly synthesized labelled proteoglycans were bound at the cell

Table 1. Distribution of newly synthesized glycosaminoglycans among intra-, peri- and extra-cellular pools

Chondrocytes were incubated with 20 μCi of [^{35}S]sulphate/ml for 4 h in medium with 1 mg of hyaluronic acid (HA)/ml, with 3 mg of bovine nasal-septum-cartilage proteoglycan (PG)/ml or without additions as control. Cells were treated with trypsin to separate peri- and intra-cellular pools (see the Experimental section). Glycosaminoglycans were determined as cetylpyridinium chloride-precipitable [^{35}S]sulphate label as described in the Experimental section. The results shown are the means for two cultures. The percentage distribution represents the percentage of label in each pool of the total amount of label in each type of culture.

	Distribution of ^{35}S label	
	(d.p.m.)	(% of total)
Medium pool		
Control	168 337	63
HA added	269 634	82
PG added	252 191	83
Pericellular pool (trypsin-releasable)		
Control	90 031	34
HA added	52 412	16
PG added	44 945	15
Intracellular pool (trypsin-resistant)		
Control	8 222	3
HA added	6 920	2
PG added	5 591	2

surface and could be released by trypsin digestion (Table 1). Both hyaluronic acid and proteoglycan monomer when added in a large excess competed for binding of a portion of the endogenously synthesized proteoglycans to the cells, i.e. cell-bound proteoglycans were decreased by half to 15% of the total and those in the medium increased correspondingly (Table 1).

Kinetics of proteoglycan pools, with and without competition with hyaluronic acid

A pulse-chase experiment was performed. The cells, with or without added hyaluronic acid in the medium, were labelled with a short pulse of [^{35}S]sulphate and then chased without label for different times. There was no increase in the total amount of ^{35}S -labelled macromolecules during the chase, indicating that the removal of free [^{35}S]sulphate was efficient (Fig. 1a). In control cultures without hyaluronic acid (Fig. 1b) the medium pool had already after 10 min reached a maximum of 68% of the total macromolecular radioactivity, and this then slowly decreased to 39% after 90 min, with a concomitant increase of the pericellular pool. This pattern was reproduced in several experiments, although the relative proportions of label between pools varied somewhat. In other cultures with hyaluronic acid in the medium (Fig. 1c) the medium pool reached a plateau level of about 75% of the proteoglycans after 10 min, while the pericellular pool contained a rather constant amount, 12–19% of the labelled macromolecules, throughout the experiment. It appears that in cultures without added hyaluronic acid most of the proteoglycans are first exported into the medium and that only later a portion binds to the cell surface. This binding was blocked by the addition of hyaluronic acid to the medium. As has been

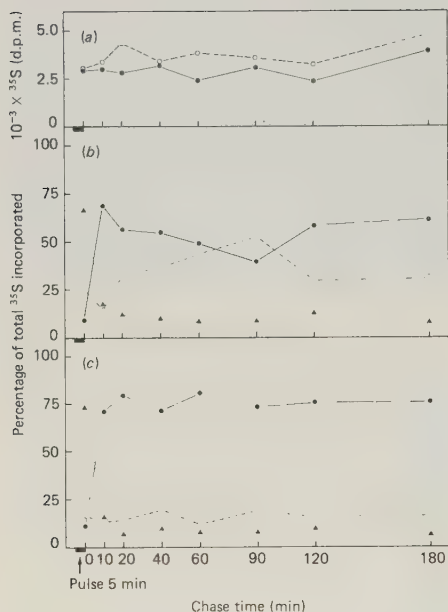


Fig. 1. Changes in distribution of labelled glycosaminoglycans with time after short labelling in cultures with or without added hyaluronic acid

The cells were incubated with $10 \mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate/ml for 5 min in medium with 1 mg of hyaluronic acid/ml (c) or without addition as control (b). A chase was then performed for the indicated times by a change to non-radioactive medium with or without hyaluronic acid respectively, in both cases containing an excess of 3 mM- Na_2SO_4 . Analysis was performed as described in the Experimental section. (a) Total amount of $[^{35}\text{S}]$ sulphate incorporated in glycosaminoglycans: —, control without hyaluronic acid in the medium; ----, hyaluronic acid in the medium. (b) Percentage distribution of labelled macromolecules in control cultures: —, medium pool; --, pericellular pool; ·····, intracellular pool. (c) percentage distribution of labelled macromolecules in cultures with hyaluronic acid in the medium: —, medium pool; ----, pericellular pool; ·····, intracellular pool.

shown previously (Sommarin & Heinegård, 1983), the added hyaluronic acid competes with that at the cell surface for binding of proteoglycans. Notably, however, the pericellular pool of proteoglycans remains constant and surprisingly large in the presence of hyaluronic acid. The molecules in this pool appear to become directly bound at the cell membrane, possibly being structurally different and intercalated in the cell membrane. We therefore proceeded to isolate and characterize these cell-bound proteoglycans.

Analytical Sephacryl S-500 gel chromatography of cell extracts

Chondrocytes were labelled with $[^{35}\text{S}]$ sulphate and $[^3\text{H}]$ leucine during 4 h and extracted with CHAPS/guani-

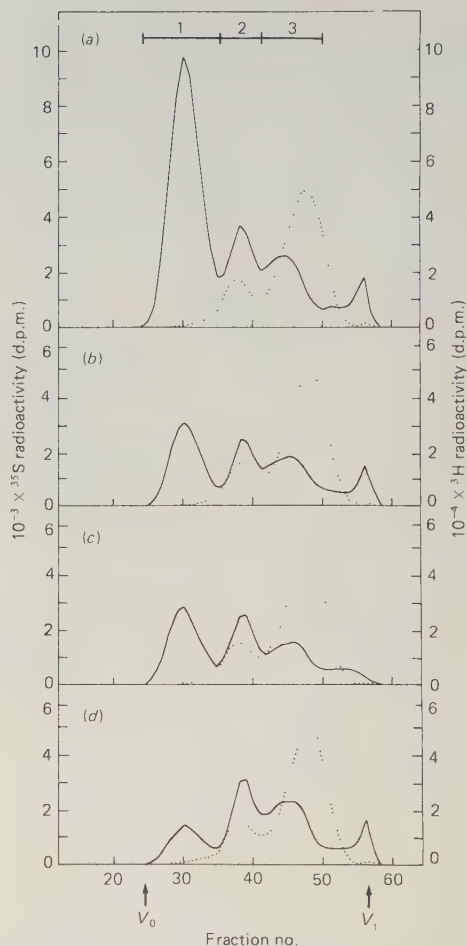


Fig. 2. Analytical Sephacryl S-500 gel chromatography of cell extracts

Chondrocytes were incubated with $40 \mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml and $10 \mu\text{Ci}$ of $[^{35}\text{S}]$ sulphate/ml for the times indicated. The cells were washed once and then extracted with a combination of detergent and guanidium chloride. Cell extracts were chromatographed on a Sephacryl S-500 column eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate buffer, pH 5.8. —, ^{35}S radioactivity; ·····, ^3H radioactivity. Details are given in the Experimental section. (a) 4 h pulse. Bars indicate the fractions chosen for calculation of proportions of the peaks. (b) Hyaluronic acid (1 mg/ml) added, 4 h pulse. (c) Hyaluronic acid (1 mg/ml) added, 4 h pulse followed by 20 min chase by adding Na_2SO_4 to a concentration of 3 mM and leucine to 2 mM. (d) 4 h pulse with 0.1 TRU/ml of *Sireptomyces* hyaluronidase and $20 \mu\text{g}$ of ovomucoid/ml (as proteinase inhibitor) present in the medium as described previously (Sommarin & Heinegård, 1983). In this and other Figures V_0 and V_1 represent the void volume and total volume respectively.

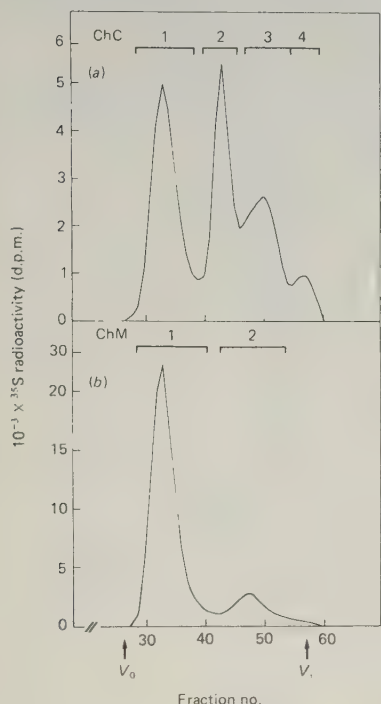


Fig. 3. Preparative Sephacryl S-500 gel chromatography of all extract and medium

Chondrocytes were labelled with [^{35}S]sulphate in medium containing hyaluronic acid (1 mg/ml) for 4 h followed by a chase in fresh medium without radiolabel for 20 min. Proteoglycans in cell extracts and medium were purified by DEAE-cellulose ion-exchange chromatography and chromatographed on a Sephacryl S-500 column eluted with the same buffer as before. (a) Cell extract. (b) Medium. Bars indicate pooled fractions.



Fig. 4. Fluorography of agarose/polyacrylamide-gel electrophoresis of pooled fractions

Samples were precipitated with ethanol and electrophoresed on agarose/polyacrylamide gels as described in the experimental section. Lane a, pool ChM1; lane b, pool ChC1; lane c, pool ChC2; lane d, pool ChC3; lane e, pool ChC4; lane f, pool ChM1; lane g, pool ChM2; lane h, pool ChC3. The two outer lanes show the carrier bovine articular-cartilage proteoglycans.

macromolecules at the cell surface, however, was almost the same with and without the chase (6% reduction only) (Figs 2b and 2c). Consequently most of the [^{35}S]sulphate-labelled material, identified in the cell extracts, was bound at the cell surface. The cell-associated [^{35}S]sulphate label was decreased by 56% when binding of the large proteoglycans appearing in the first peak was prevented by digestion of hyaluronic acid at the cell surface by *Sireptomyces* hyaluronidase (Fig. 2d). No changes were seen in [^3H]leucine-labelled material, which probably largely represents proteins.

Preparation of [^{35}S]sulphate-labelled proteoglycans from cell extracts and medium

Cells were pulse-labelled in medium with added hyaluronic acid and [^{35}S]sulphate for 4 h followed by a 20 min chase to allow newly sulphated molecules to be exported. Proteoglycans bound to the cells were extracted with CHAPS/guanidinium chloride. An initial ion-exchange chromatography on DEAE-cellulose eluted with solutions containing 6 M-urea removed approx. 90% of the protein material in the cell extracts. Also, the proteoglycans in the medium were purified by using DEAE-cellulose chromatography. The proteoglycan fractions were then chromatographed on Sephacryl S-500 (Fig. 3). The peaks were pooled and are referred to by the numbers indicated in Fig. 3.

Agarose/polyacrylamide-gel electrophoresis

On agarose/polyacrylamide-gel electrophoresis of pooled fractions (Fig. 4) the large components from the cell extract, ChC1, and medium, ChM1, both migrated as the slowest band of the carrier bovine articular-cartilage proteoglycan. Interestingly these cultured chondrocytes appear to synthesize only one type of large aggregating proteoglycan, whereas two have been identified in extracts of cartilage (for references see Heinegård *et al.*,

dinium chloride as described in the Experimental section. Samples of extracts were chromatographed on Sephacryl S-500. Those from the culture where no hyaluronic acid had been added to the medium (Fig. 2a) contained a predominating peak of ^{35}S label eluting at the same position as large cartilage proteoglycans. This peak represented 60% of the [^{35}S]sulphate-labelled macromolecules (the amount of radioactivity in the total volume of the column was not included in the calculation of percentage distribution since it contained some free [^{35}S]sulphate). Two smaller, more-retarded, peaks represented 19% each of ^{35}S label. When hyaluronic acid was included in the medium, the total amount [^{35}S]sulphate-labelled material recovered from the cells was reduced by about half (53%), owing to a decrease in the first peak (Fig. 2b). In a control experiment, the 4 h pulse was followed by a 20 min chase to allow export of newly synthesized intracellular [^{35}S]sulphate-labelled material (Fig. 2c). The total amount of [^{35}S]sulphate-labelled

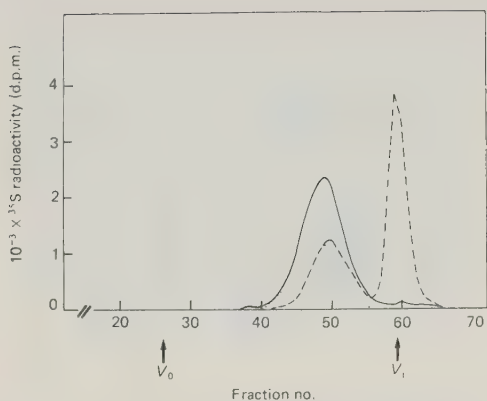


Fig. 5. Sephacryl S-500 chromatography of dermatan sulphate proteoglycans and heparan sulphate proteoglycans in fraction ChC3

Samples of fraction ChC3 were prepared for gel chromatography by dialysis against 0.1 M-Tris/0.1 M-sodium acetate buffer, pH 8. One half was digested with 0.05 unit of chondroitinase ABC for 4 h at 37 °C, and the other half was incubated in parallel without enzyme, as a control. Gel chromatography was performed on a Sephacryl S-500 column (145 cm $\times$ 0.6 cm) eluted as indicated in Fig. 2 legend. —, Control; ----, chondroitinase ABC-digested sample representing heparan sulphate proteoglycan; curve representing the dermatan sulphate proteoglycan, constructed by subtracting the chondroitinase ABC-resistant material from the control (negative values for fractions 56–65 omitted).

1985a). The cells used in the present study, however, were derived from calf articular cartilage, and it has been observed that cartilage from foetus and calf contains only the slowly migrating proteoglycan (Inerot & Heinegård, 1983).

Proteoglycans in fraction ChC2 migrated much more slowly than any of the others. This fraction also contained a less-pronounced faster-moving component at a position corresponding to the smaller proteoglycans in fraction ChC3, probably from admixture of small proteoglycans of the type found in fraction ChC3. The third pool from the cell extract, ChC3, appeared as a broad band, at the position of small cartilage proteoglycans (Heinegård *et al.*, 1985b). The small proteoglycan from the medium, ChM2, produced a distinct band of mobility similar to that of ChC3. The smallest component in the cell extract, ChC4, could not be detected, possibly because of loss of these molecules during immersion of the gel in the aqueous scintillator.

Size of different proteoglycans in fraction ChC3

Since fraction ChC3 appears to contain at least one heparan sulphate-containing and one dermatan sulphate-containing proteoglycan (see below), information on their size distributions was studied by chromatography of chondroitinase ABC-treated fraction ChC3. The resistant heparan sulphate-containing proteoglycan was eluted somewhat more retarded on Sephacryl S-500 (Fig. 5,

Table 2. Glycosaminoglycan composition [35 S]sulphate-labelled material in proteoglycan fractions

Free glycosaminoglycan chains from the pooled fractions were tested for the presence of dermatan sulphate, chondroitin sulphate and heparan sulphate by degradation with chondroitinase AC, chondroitinase ABC and HNO_2 respectively. Details are given in the Experimental section.

Pooled fraction	Percentage degraded by treatment with		
	Chondroitinase AC	Chondroitinase ABC	HNO_2
ChC1	96.8	97.1	0.1
ChC2	92.3	95.9	0.9
ChC3	60.7	69.0	23.2
ChC4	47.7	57.2	36.2
ChM1	98.0	98.2	0.5
ChM2	73.0	94.9	4.0

broken line) than the dermatan sulphate proteoglycans (Fig. 5, dotted line).

Glycosaminoglycan composition of pooled fractions

Glycosaminoglycan chains were liberated from the proteoglycans by alkaline treatment and analysed for the presence of chondroitin sulphate and heparan sulphate by susceptibility to degradation with chondroitinase AC, chondroitinase ABC and HNO_2 respectively (Table 2). Glycosaminoglycans present in fractions ChC1, ChC2 and ChM1 were almost completely depolymerized by chondroitinase AC treatment, indicating that they contained only chondroitin sulphate. The two fractions of the cell extract containing smaller molecules, i.e. ChC3 and ChC4, were only partially depolymerized, i.e. 60–70% and 47–57% respectively of the label was recovered as disaccharides. They were depolymerized to a somewhat larger extent by chondroitinase ABC, although depolymerization was not complete. The difference between the results with the two enzymes indicates that the materials in these two fractions contain a form of dermatan sulphate with a low degree of epimerization, with up to 10% of the disaccharides containing iduronic acid. A portion (25–35%) of the total material in both fractions, corresponding to that resistant to chondroitinase ABC, could be depolymerized by treatment with HNO_2 . This material represents heparan sulphate. Digestion of fraction ChM2 with chondroitinase AC gave 73% depolymerization, whereas chondroitinase ABC gave almost complete depolymerization, indicating this fraction contains dermatan sulphate, probably with an iduronic acid content of some 25%.

Size of glycosaminoglycan chains

The size of free glycosaminoglycan chains from the different preparations were determined by gel chromatography on a Superose 6B column calibrated with chondroitin sulphate chains of known M_r values (Fig. 6). Comparison of glycosaminoglycan chains from fractions ChC1 and ChM1 shows that they are of similar size. The chondroitin sulphate chains from fraction ChC2 are larger than those from fractions ChC1 and ChM1, indicating the different nature of these proteoglycans. The largest chains were from the small proteoglycan in the

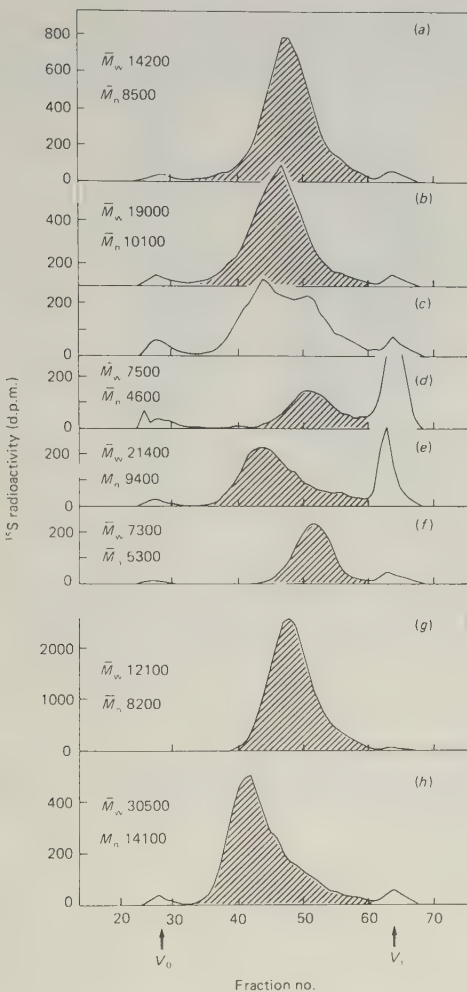


Fig. 6. Size determination of labelled glycosaminoglycan chains by gel chromatography on Superose 6B

Free glycosaminoglycan chains were prepared from pooled fractions and chromatographed on a Superose column calibrated with chondroitin sulphate standards. Details are given in the Experimental section. Hatched areas indicate fraction chosen for calculation of M_w (weight-average M_r) and M_n (number-average M_r). (a) Pool ChC1; (b) pool ChC2; (c) pool ChC3; (d) pool ChC3 treated with chondroitinase ABC leaving heparan sulphate chains intact; (e) pool ChC3 treated with HNO_2 , leaving dermatan sulphate chains intact; (f) pool ChC4; (g) pool ChM1; (h) pool ChM2.

medium, ChM2, which also were very polydisperse, shown both by the chromatogram and by the difference between weight-average and number-average M_r values. The material in pool ChC3 gave a profile with two peaks

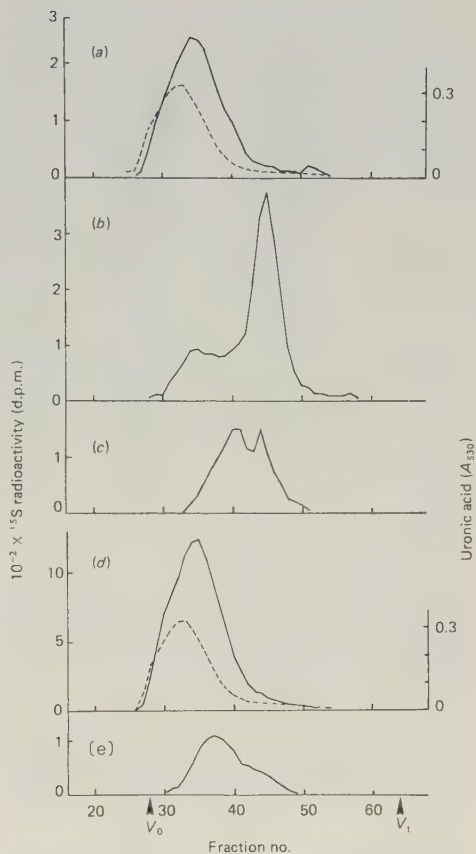


Fig. 7. Size of trypsin-digest fragments of proteoglycans determined by Superose 6B gel chromatography

Samples of pooled fractions were treated with trypsin and chromatographed on a Superose 6B column. Details are given in the Experimental section. (a) pool ChC1; (b) pool ChC2; (c) pool ChC3; (d) pool ChM1; (e) pool ChM2.

—, ^{35}S radioactivity; ---, A_{530} .

(Fig. 6c), probably representing dermatan sulphate and heparan sulphate. This material was treated with chondroitinase ABC (Fig. 6d) or with HNO_2 (Fig. 6e) before gel chromatography to depolymerize selectively dermatan sulphate and heparan sulphate respectively. The heparan sulphate chains (Fig. 6d) were small, of average M_r only 7500. The dermatan sulphate chains (Fig. 6e) were smaller than those from the small proteoglycan in the medium. They were also polydisperse, with similar differences in weight-average and number-average M_r values as those of fraction ChM2. The pool with the smallest material, ChC4, contained only low- M_r material, which did not change elution position after chondroitinase ABC treatment (results not shown).

Some caution should be exerted when interpreting the

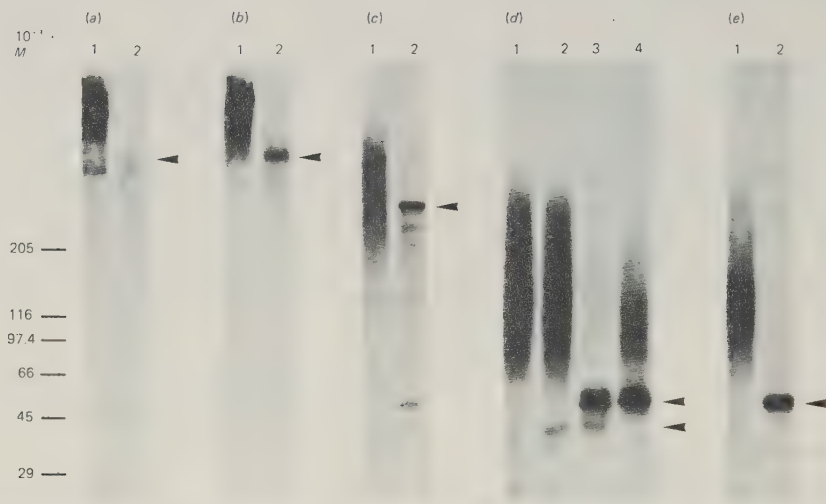


Fig. 8. Detection of proteoglycan core protein by SDS/polyacrylamide-gel electrophoresis of [^3H]leucine/[^{35}S]sulphate-labelled proteoglycan populations

Samples were prepared and electrophoresed on polyacrylamide gradient gels ($3\text{--}10^4$) as described in the Experimental section. (a) Pool ChC1: lane 1, untreated; lane 2, chondroitinase ABC-digested. (b) Pool ChM1: lanes as in (a). (c) Pool ChC2: lanes as in (a). (d) Pool ChC3: lane 1, untreated; lane 2, heparitinase-digested; lane 3, heparitinase- and chondroitinase ABC-digested; lane 4, chondroitinase ABC-digested. (e) Pool ChM2: lanes as in (a). Arrowheads indicate position of core protein. M_r values of reference proteins are indicated.

absolute values for the M_r values, since the values are all related to chondroitin sulphate standards, and dermatan sulphate and heparan sulphate may behave somewhat differently on the gel-permeation column.

Size of fragments produced by trypsin digestion

The chondroitin sulphate chains in the major, large, proteoglycans from cartilage appear to be clustered along the core protein. Trypsin treatment yields peptide fragments, probably corresponding to these clusters, each containing several chains (Heinegård & Hascall, 1974). It is possible that the enzyme has access only to sites surrounding the clustered chondroitin sulphate chains and that the size of the fragments produced will give information on the distribution of glycosaminoglycans along a proteoglycan protein core. The pooled fractions were therefore treated with trypsin and chromatographed on Superose 6B to reveal any differences in the distribution of glycosaminoglycans between proteoglycans (Fig. 7). On comparing profiles with that of the added bovine nasal-septum-cartilage proteoglycan carrier, only fractions ChC1 and ChM1 appear to have similarly clustered chains (Figs. 7a and 7d). The fragments, however, are somewhat smaller than those from the carrier, perhaps depending on the smaller chondroitin sulphate chains (Heinegård *et al.*, 1985a). The second pool from the cell extracts, ChC2, showed a predominant peak of size similar to that of the free chains in this fraction. It also contained a smaller leading shoulder with molecules of the size similar to that of the trypsin fragments from fractions ChC1 and ChC3. This material

probably represents admixture of these fractions. The other pools, ChC3 and ChM2, gave fragments rather similar in size to the free chains (Figs. 7c and 7e). The last pool from cell extracts was not analysed.

Identification of core proteins

An attempt was made to identify core proteins of the different proteoglycans by SDS/polyacrylamide-gel electrophoresis. Proteoglycan preparations from cells labelled with both [^3H]leucine and [^{35}S]sulphate were used. Although the preparations contained some contaminating proteins, core proteins could be identified after enzymic removal of glycosaminoglycan chains (Fig. 8). The large proteoglycans, ChC1 and ChM1, appeared to have large core proteins of similar size (Fig. 8a, lane 2, and Fig. 8b, lane 2). Fraction ChC2 contained a smaller core protein (Fig. 8c, lane 2) with an apparent M_r of about 300000. In both fractions ChC3 and ChM2 major core proteins of M_r about 50000 were found after removal of dermatan sulphate chains (Fig. 8d, lane 4, and Fig. 8e, lane 2). This in the same range as the core protein of small proteoglycans from cartilage (Heinegård *et al.*, 1981). Fraction ChC3, however, appears to contain more than one core protein of similar size, as indicated by the broad band seen in Fig. 8(d), lane 4. A heparan sulphate proteoglycan core protein of M_r 40000 was identified after heparitinase treatment (Fig. 8d, lane 2). It was only partially resolved from contaminating proteins, but is distinctly smaller than the core protein of the small dermatan sulphate proteoglycan. Since the results discussed above indicate that the separation between

fractions ChC2 and ChC3 was only partial, it is not surprising to find some core protein of the small dermatan sulphate proteoglycan type in fraction ChC2 and some of the large type in fraction ChC3 (Fig. 8c, lane 2, and Fig. 8d, lane 4). Fraction ChC4 was not studied.

DISCUSSION

In a previous study proteoglycans added to the medium of cultured chondrocytes were shown to bind to hyaluronic acid at the cell surface in a process similar to proteoglycan aggregate formation (Sommarin & Heinegård, 1983). In the present work, however, only about half of the cell-surface-associated endogenous [35 S]sulphate-labelled proteoglycans appeared to bind to hyaluronic acid. This suggested the existence of proteoglycans at the cell surface that were structurally different from the large aggregating proteoglycans present in cartilage matrix.

In support of this, extracts of labelled chondrocytes, when chromatographed on Sephacryl S-500, showed three distinct peaks of [35 S]sulphate-labelled proteoglycans. One peak, ChC1, contains high- M_r proteoglycans of the type considered typical for cartilage. The large cell-bound proteoglycans appeared to be identical with the large proteoglycans in the medium. A portion of the large proteoglycans only was removed from the cells by competition with hyaluronic acid or treatment with *Streptomyces* hyaluronidase. Therefore it is possible that some of these large proteoglycans were bound by some other mechanism or that they preferentially bound to hyaluronic acid at the cell surface.

In cultures with hyaluronic acid added to the medium, the cell-associated [35 S]sulphate label represents about 5% of the total in the culture. The large cell-bound proteoglycan represents 6%, whereas the large one in the medium represents 76%, of all incorporated [35 S]sulphate. Thus the major biosynthetic product of articular-cartilage chondrocytes are large aggregating proteoglycans exported into the medium.

The other proteoglycans isolated from these cultures appeared to be different. The second peak, ChC2, contained proteoglycans of a different nature from any previously described cartilage proteoglycan. This novel proteoglycan represent some 4% of the total labelled proteoglycans. It migrated more slowly than the large proteoglycans on agarose/polyacrylamide-gel electrophoresis, the chondroitin sulphate chains were not arranged in 'clusters' and the core protein was of high M_r , although smaller than those from the largest proteoglycans. On CsCl-density-gradient centrifugation it has a low buoyant density (results not shown), indicating that it has a high relative content of protein and few side chains. It is similar in size and low buoyant density to the collagenase-sensitive and disulphide-bonded proteoglycan from chick-embryo cartilage described by Noro *et al.* (1983). The proteoglycan reported here, however, was not sensitive even to prolonged collagenase treatment, and did not change position on Sepharyl S-500 chromatography after reduction and alkylation (results not shown). Another proteoglycan of similar character has been described as a specific melanoma-cell antigen (Ross *et al.*, 1983; Bumol *et al.*, 1984) and from rat ovarian granulosa-cell cultures (Yanagashita & Hascall, 1983). Interestingly, the amount of fraction ChC2 showed the largest variation between different cell preparations,

perhaps reflecting a response of the chondrocytes to environmental factors.

The third peak in the cell extracts, ChC3, contained at least two different kinds of proteoglycans. One, representing 3% of all [35 S]sulphate-labelled material, contained dermatan sulphate chains with a low degree of epimerization. The other one contained heparan sulphate chains and represented only about 1% of all the labelled material. Heparan sulphate proteoglycans showing considerable structural diversity have been isolated from a variety of sources. Examples are small (75000- M_r) proteoglycans from rat liver membranes (Oldberg *et al.*, 1979), large (300000-400000- M_r) dimeric disulphide-bonded proteoglycans from cultured fibroblasts (Cöster *et al.*, 1983) and both small (130000- M_r) and large (400000- M_r) proteoglycans from a basement-membrane-producing tumour (Fujiwara *et al.*, 1984). The chondrocyte proteoglycan appears to be similar with regard to both size of intact proteoglycan and size of core protein to the small proteoglycan from basement membrane and that from rat liver membranes. Much caution should be exerted when comparing the data, however, since different techniques have been used and the proteoglycans behave non-ideally.

The cell extract also contained low- M_r material, representing only 1% of the total label, probably a pool of intracellular material representing molecules being degraded.

The second major biosynthetic product was the small dermatan sulphate proteoglycan in the medium, representing about 9% of the labelled material. Indeed, the presence of small dermatan sulphate-containing proteoglycans in bovine articular cartilage has been shown by the studies made by Rosenberg *et al.* (1985). The small proteoglycan in the medium may be related to the small dermatan sulphate proteoglycan at the cell surface. Even though their glycosaminoglycan compositions and chain sizes differ, the proteoglycans are of approximately the same size as are their core proteins.

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Metabolism of cell-associated
proteoglycans in cultures of articular-
cartilage chondrocytes

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SYNOPSIS

Metabolism of [^{35}S]-sulphate labelled cell-associated proteoglycans was studied in monolayer cultures of bovine articular cartilage chondrocytes. Cell surface proteoglycans were labelled for 22 hours and their elimination was followed during eight days of subsequent chase. Classes of proteoglycans in cell layer extract and medium were separated and quantitated by CsCl density-gradient centrifugation followed by gel permeation chromatography.

Large aggregating proteoglycans were most rapidly released into the medium with a half-life for the cell-associated molecules of about 4 days (95h). A proteoglycan of intermediate size, previously only identified at the cell surface, was released into the medium with $t_{1/2}$ of approximately 8 days. However, only 44% of this proteoglycan could be accounted for in the medium indicating possible internalization of the molecule and degradation by the cells. Small proteoglycans, i.e. one with dermatan sulphate and one with heparan sulphate side chains, were eliminated from the cell surface also with a $t_{1/2}$ of about 8 days. At least a proportion of these proteoglycans appear to be released into the medium.

INTRODUCTION

Chondrocytes appear to have the ability to monitor properties of their surrounding matrix. Thus matrix components added to cultured cells may influence their activity as exemplified by in vitro experiments where the addition of proteoglycans or hyaluronic acid to cultured chondrocytes modulates proteoglycan synthesis (Nevo & Dorfman, 1972; Wiebkin & Muir, 1973; Handley & Lowther, 1976, 1977; Lowther & Handley, 1979). Changes in mechanical load, either on cartilage in joints or on high density cultures of chondrocytes, also affect proteoglycan synthesis (Palmoski et al., 1979, 1980; van Kampen et al., 1985). It is likely that these effects are mediated via interactions at the cell surface but the nature of such interactions is largely unknown. Furthermore, collagen type II can bind to a receptor molecule that has been isolated from chondrocytes (Mollenhauer et al., 1983, 1984). Large aggregating proteoglycans are able to bind to hyaluronate at the cell surface of cultured chondrocytes (Sommarin & Heinegård, 1983).

Further studies of the cell-associated proteoglycans in chondrocyte cultures showed in addition to large proteoglycans bound to hyaluronic acid, three other types of proteoglycans were present (Sommarin & Heinegård, 1986). These were an intermediate size chondroitin sulphate proteoglycan and two types of small proteoglycan, i.e. one dermatan sulphate proteoglycan and one heparan sulphate proteoglycan. In the present investigation the metabolic fate of the cell-associated proteoglycans is studied in order to obtain information whether those at the cell surface are internalized and degraded by the chondrocyte or become released to the medium indicating that they are deposited in the cartilage matrix. The data presented in this paper show different turnover rates of the cell-associated proteoglycans and also indicating that both the large aggregating and the intermediate size proteoglycans are released into the medium.

EXPERIMENTAL

Materials

Ham's F12 medium (GIBCO, Paisley, Renfrewshire, Scotland) supplemented with 20 ul of 50X essential amino acids (for Eagle's minimum essential medium; Flow Laboratories, Bethesda, MD,

U.S.A.)/ml, 20 ug of dextran sulphate (Pharmacia Fine Chemicals, Uppsala, Sweden)/ml, 50 i.u. each of penicillin and streptomycin (Flow Laboratories)/ml and buffered with 20mM Hepes (Sigma Chemical Co., St Louis, MO, U.S.A.), pH 7.4, was used for spinner culture. Bacteriological Petri dishes (85 mm diameter) were from Nunc AS (Roskilde, Denmark).

Cell culture and pulse-chase procedure

Articular cartilage chondrocytes were prepared from the fetlock joint of calves as described previously (Sommarin & Heinegård, 1983). Chondrocytes were cultured in serum free medium at all times. The chondrocytes were kept in spinner culture for 24 hours after isolation to allow reconstitution of the cell surface and remove damaged cells. Cells were collected, washed and plated on 85 mm diameter bacteriological petri dishes at a density of 75000 cells/cm² in 10ml of Ham's F12 medium supplemented with 25 i.u. of penicillin/ml and buffered with 20 mM Hepes, pH 7.4 referred to as (Ham's F12H). The cultures were kept at 37°C in humidified air.

After 24 hours in monolayer culture the medium was replaced with fresh Ham's F12H containing 50 uCi [³⁵S]-sulphate/ml. Cultures were labelled for 22 hours. The medium was removed and the cell layer was carefully rinsed three times with chase medium (Ham's F12H without isotope and the sulphate concentration raised to 1 mM) and fresh chase medium. Chase medium was changed every two days.

Medium was removed at indicated times and the cell layer carefully rinsed with 4 x 0,5 ml fresh medium. The rinse was pooled with the medium and centrifuged to remove any cells. These represented less than 2% of all cells in the culture. After one week of culture viability was above 98% as judged by Trypan blue exclusion test. Cells and media were kept at -60°C until analysis.

Extraction of cells

The cell layer was extracted with 2 ml of 8% CHAPS/0.1 M sodium acetate buffer, pH 5.8 also containing the proteinase inhibitors EDTA (20 mM), 6-aminohexanoic acid (0,2 M), benzamidinium chloride (10 mM), N-ethylmaleimide (10 mM) and phenylmethanesulphonyl fluoride (2 mM) at 40°C on a rotary shaker. After 15 minutes an equal volume of 8 M

guanidinium chloride was added and extraction continued for 45 minutes. Non-soluble material in both cell layer extract and medium was removed by centrifugation at 105000 g for one hour at 4°C in a 70.1 Ti angle rotor (Beckman Instruments, Palo Alto, CA, USA).

Fractionation of proteoglycans

Samples of cell layer extract and medium were taken to CsCl-density gradient centrifugation to fractionate proteoglycans. Medium was adjusted to 4 M guanidinium chloride, 0.1 % Triton X-100 and 1/10 concentration of the proteinase inhibitors listed above. The starting density was adjusted to 1.5 g/ml by addition of solid CsCl. Centrifugation was performed in Quickseal tubes (Beckman Instruments) at 40 000 rpm, 15°C for 65 hours in a 70.1 Ti angle rotor. The tubes were divided, with the aid of a Beckman tube slicer, into two parts. The upper 1/6, had an average density of 1.35-1.38 g/ml and the remaining bottom 5/6 had an average density of 1.52-1.55 g/ml.

Samples of fractions from the CsCl-density gradient were analyzed by gel permeation chromatography on serially coupled columns of Sephacryl S-500 (0.65x47 cm) and Superose 6B (0.65x145 cm)(Pharmacia Fine Chemicals) in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 and 0.5% Triton X-100. The first column gives good separation of large proteoglycan species and the second improves separation of the intermediate size proteoglycan from low molecular weight proteoglycans. Fractions of 0.9ml were collected and assayed for radioactivity.

RESULTS

Pulse-chase experiment

Cultures were incubated for 22 hours with 50uCi [³⁵S]sulphate/ml of medium. This medium was replaced with non-radioactive medium containing a supplement of sodium sulphate to prevent incorporation of any residual free radioactive sulphate or such produced by degradation of glycosaminoglycans.

Separation and quantitation of proteoglycan populations in cell layer extract and medium.

After the 22 hour labelling period large aggregating proteoglycans constituted 90% of the labelled macromolecules in the medium and 84% of the labelled macromolecules at the cell layer. Preliminary experiments showed that separation on gel permeation chromatography of these large proteoglycans from some of the minor proteoglycan constituents was only partial. The large, highly polyanionic proteoglycans were therefore separated from the other proteoglycans by CsCl-density gradient centrifugation. A high starting density was used to shift all the low density proteoglycans to the top of the tube. The gradients were divided into two parts 1/6 of the length from the top at a density of about 1.40g/ml. Proteoglycan populations were quantitated by gel permeation chromatography of samples from the top and the bottom fractions of the gradient respectively. Examples of the separations of the cell extracts, Fig. 1, and medium, Fig. 2, show that all large, high density proteoglycans were recovered in the bottom 5/6 of gradient. On the other hand all of the intermediate size proteoglycan were recovered in the top fraction, Fig. 1a.

The small proteoglycans, representing a mixture of dermatan sulphate and heparan sulphate proteoglycans, chromatographing most retarded on the column were surprisingly polydisperse with respect to buoyant density. Although a major proportion (about 75% at the onset of chase) of those bound to the cells were recovered in the top, low density fraction (Fig.1 a, c), small proteoglycans were recovered in the bottom, high density fraction also, (Fig.1 b, d). The quantitation of the latter material, however, is approximate, since there was considerable trailing of large proteoglycans.

A large proportion (some 75%) of the small medium proteoglycans was recovered in the bottom fraction, but again values are approximate due to trailing of the large proteoglycans (Fig.2). It is possible that a portion of these small proteoglycan actually represents chondroitin sulphate peptides derived by degradation of the large proteoglycans. It has not been possible to study this question further.

Turnover of proteoglycans

Large cell-associated proteoglycans. The large proteoglycans showed the most rapid turnover, those at the cell surface decreasing from 2.6×10^6 to 0.4×10^6 dpm per million cells, Fig. 3a, i.e. an 85% decrease during the eight days of culture. These proteoglycans appear not to be internalized and degraded by the chondrocytes since those lost from the cells were almost quantitatively (77%) recovered in the medium, Fig. 4a.

The time course of release from the cells had a biphasic appearance with rapid elimination during the first two days followed by a phase with slow release until the end of the experiment. All cell-associated proteoglycans showed a similar biphasic time course, Fig. 3. The half life of the large proteoglycans calculated for the rapid phase from data during the first seven hour chase, was about 8h, while proteoglycan half-life for the slow release phase of the large proteoglycans fitted well to first order kinetics with a calculated half-life of 3 days and 23 hours, Fig.3a.

Large proteoglycans released from the cells (Fig. 2d) appeared to be more polydisperse having a trailing peak on the gel chromatogram, than proteoglycans directly released into the medium during labelling (Fig.2b). They were also more polydisperse than those present at the cell surface both at the beginning (Fig. 1b) and at the end (Fig. 1d) of the experiment.

Intermediate size proteoglycan. The intermediate size proteoglycan had a similar biphasic, but slower turnover with a 65% reduction of the amount at the cell surface during the length of the experiment. This proteoglycan was also released into the medium where it could be identified, (Fig2 a, c). However, only 44% of those present at the beginning of the chase could be accounted for in the medium. This may either be due to low recovery or to internalization and degradation of this proteoglycan. The two phases of elimination of these proteoglycans from the cell surface correspond to 4h and 194h (~8days), respectively, i.e. the latter more than twice as long as that of the large proteoglycans. The intermediate size proteoglycan released into the medium consistently eluted slightly more retarded on gel permeation chromatography than those remaining at the cell surface, compare fig. 1c and 2c. This may indicate release by limited proteolytic cleavage. However, the conditions of chromatography with different

amounts of carrier material in cell layer extract and medium or different proportions of proteoglycans may influence elution positions.

Small proteoglycans. Only data from the top fraction of the CsCl gradient are evaluated since values for the higher buoyancy component, i.e. the bottom fraction, may represent mixtures of intact and fragmented proteoglycans. A 76% decrease of these proteoglycans was seen in the cell layer at the end of the experiment. Only 15% of the lost molecules could be accounted for in the medium (top fraction). Thus it is likely that a substantial proportion of these proteoglycans for the most part are degraded by the cells, unless as described above they are partially cleaved by proteolysis when released from the cells and the higher buoyant density fragment is recovered in the bottom fraction. Unfortunately the trailing of the prominent large proteoglycans hamper further attempts to quantitate the small proteoglycans present in the high buoyant density fraction (see Fig.2d).

The small low buoyant density cell associated proteoglycans showed the same biphasic elimination with a time course of elimination similar to that of the intermediate size proteoglycan. A half-life of 208 hours (almost 9 days) was calculated for the slow phase (using values from day 2 to 8) while the rapid phase had an estimated half life of 6h.

A shift in the size of the proteoglycans at the cell surface can be seen. At the beginning of chase the small proteoglycans chromatograph as a broad and continuous spectrum of sizes, Fig.1a. This is gradually changed with time, such that the smaller of the molecules become more prominent.

This group of small proteoglycans have previously been shown to contain two kinds, with different core proteins and glycosaminoglycan side chains of dermatan sulphate and of heparan sulphate, respectively (Sommarin & Heinegård, 1986). Analysis for the presence of these proteoglycans was done by determination of sensitivity to digestion with chondroitinase AC/ABC to detect chondroitin/dermatan sulphate as described earlier (Sommarin & Heinegård, 1986). At the beginning of the chase, 35-40% of the label in small proteoglycans was resistant to chondroitinase ABC treatment indicating presence of heparan sulphate. The chondroitinase ABC resistant material decreased to about 20% of all small proteoglycans at day 2 and thereafter remained constant until day 8. These

results indicate that the heparan sulphate proteoglycan is more rapidly eliminated and that the proteoglycans at the cell surface on day 2 is almost exclusively dermatan or chondroitin sulphate.

DISCUSSION

In preliminary experiments on proteoglycan turnover in suspension cultures it was found that some of the cell-associated proteoglycans had a slow turnover. To study these proteoglycans we changed the culture from suspension to monolayer on hydrophobic substrate allowing better conditions for long term experiments. It should be stressed that conditions are similar for the two cultures, since chondrocytes in serum free medium readily attach to hydrophobic plastic, but do not spread, not even during long term culture (Björnsson & Heinegård, 1981). Although the cells deposit proteoglycans in a pericellular matrix this is limited and similar in nature to the cell-associated pool of proteoglycans in suspension culture as shown by the present experiments. It does neither form a continuous matrix between cells nor between cells and substrate as they do in monolayer cultures on hydrophilic substrates commonly used for cell culture (Björnsson & Heinegård, 1981).

All proteoglycan species followed the same general pattern of elimination from the cells with one phase of rapid removal markedly observed during the first one or two days and one phase with slower removal. The number of time points available does not allow detailed analysis of the rapid phase, but $t_{1/2}$ for rapid removal of all proteoglycans range 4 to 8h.

In the slower phase of proteoglycan removal, those belonging to the group of large aggregating proteoglycans showed about twice as fast elimination from the cell surface as the other cell-associated proteoglycans. These large proteoglycans were almost quantitatively released into the medium indicating little or no internalization and degradation by the chondrocytes. That chondrocytes apparently do not internalize and degrade large proteoglycans has been reported earlier (von Figura et al., 1980; Sommarin & Heinegård, 1983).

Large proteoglycans appear to be released from the cell surface both as aggregates with hyaluronic acid or as monomers (unpublished). The

presented data do, however, not exclude partial proteolysis resulting in release of a proportion of large proteoglycans not capable of interacting with hyaluronic acid. The somewhat increased polydispersity of proteoglycans released to the medium may actually be taken to indicate proteolysis. In cultures of cartilage sections the normal route of proteoglycan turnover appears to result from a proteolytic cleavage that releases the major portion of the proteoglycan from hyaluronic acid (Ratcliffe et al., 1986).

In an earlier study, comparison of cell-associated and medium proteoglycans showed an intermediate size proteoglycan which was only detected at the cell surface (Sommarin & Heinegård, 1986). In the present study it could be shown that about half the amount of this proteoglycan present at the beginning of the chase was released into the medium where the molecule could be identified. The turnover of this proteoglycan was slower than that of the large proteoglycan indicating different pathways of elimination. In view of the release of this intermediate size proteoglycan into the medium it may be present in the matrix of intact cartilage. It is likely that it has hitherto been overlooked, this being a result of its very low buoyant density. Furthermore it may be present in cartilage matrix in very small amounts not easily extractable.

The small cell-associated proteoglycans showed the same elimination rate as those of intermediate size. Only a minor fraction of the small proteoglycans were, however, found in the medium indicating extensive internalization and degradation. Because of the problems to account for all small proteoglycans the data should be interpreted cautiously. The presented results may, however, indicate altered properties of the small proteoglycans when released from the cells to the matrix, resulting in an increased buoyance upon CsCl-centrifugation.

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FIGURE LEGENDS

Figure 1. *Separation of cell layer proteoglycans by gel permeation chromatography of fractions prepared by CsCl density gradient centrifugation.*

Samples (0.25 ml) of top or bottom fractions from CsCl density gradient centrifugation of cell layer extracts were applied to serially coupled columns of Sephacryl S-500 and Superose 6 in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, 0.5% Triton X-100. Fractions of 0.9 ml were collected and 0.45 ml portions were assayed for radioactivity. Only the first and last time points of the pulse-chase experiment are shown. Bars indicate the fractions used to calculate the amount of proteoglycan of each type.

- a) Top fraction of cell layers extract directly after 22 h of pulse (before chase)
- b) Bottom fraction of cell layer extract directly after 22 h of pulse
- c) Top fraction of cell layer extract after 8 days of chase
- d) Bottom fraction of cell layer extract after 8 days of chase

Figure 2. *Separation of medium proteoglycans by gel permeation chromatography of fractions prepared by CsCl density gradient centrifugation.*

Conditions were the same as in Fig. 1 except that 0.78 ml samples were applied and 0.2 ml of each fraction were assayed for radioactivity.

- a) Top fraction of the 22 h labelling medium
- b) Bottom fraction of the 22h labelling medium
- c) Top fraction of chase medium from day 6 to 8
- d) Bottom fraction of chase medium from day 6 to 8

Figure 3. *Proteoglycans in the cell layer at different chase times.*

Fractions indicated by bars in Fig. 1 a, b were used to calculate the proportion of each proteoglycans class in fractions from the CsCl density gradient centrifugation. To minimize experimental variation in application of sample and amount of each fraction assayed for radioactivity the relative proportion of each proteoglycan class was multiplied with the total amount of label separately determined in samples of the respective CsCl density gradient fraction. Values at the one day chase time point deviate from those expected in relation to surrounding values and are therefore not included in calculations.

a) Large proteoglycan

b) Intermediate size proteoglycan (○) and small proteoglycans (■)

Figure 4. *Proteoglycans accumulated in the medium at different chase times.*

Values are calculated as described for cell layer extract. Values at zero day chase, represent proteoglycans directly exported into the medium during the 22 hour pulse labelling. Thus they do not represent proteoglycans released from the cell layer pool in contrast to all other values. The data shown represent accumulated values for each proteoglycan.

a) Large proteoglycan

b) Intermediate size proteoglycan (○) and small proteoglycan (■)

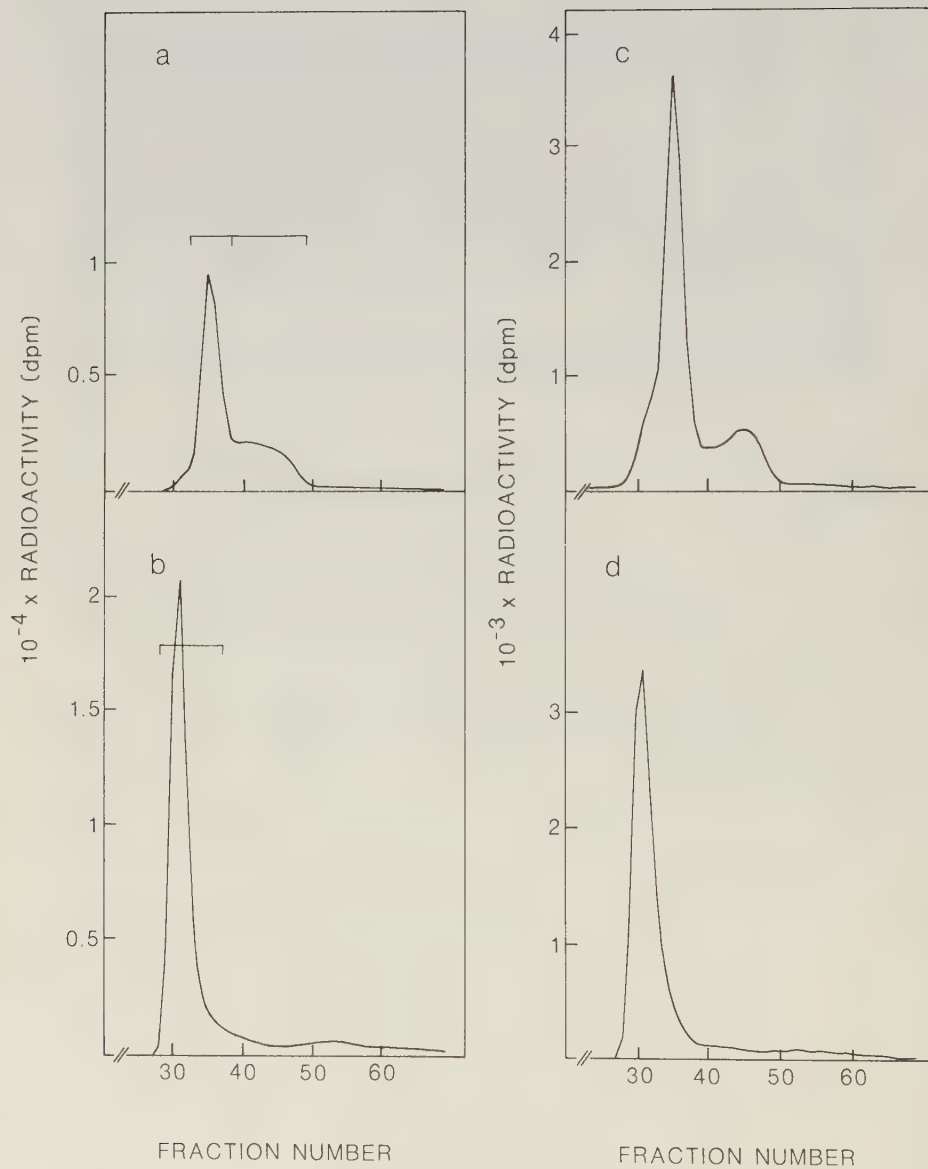


Figure 1.

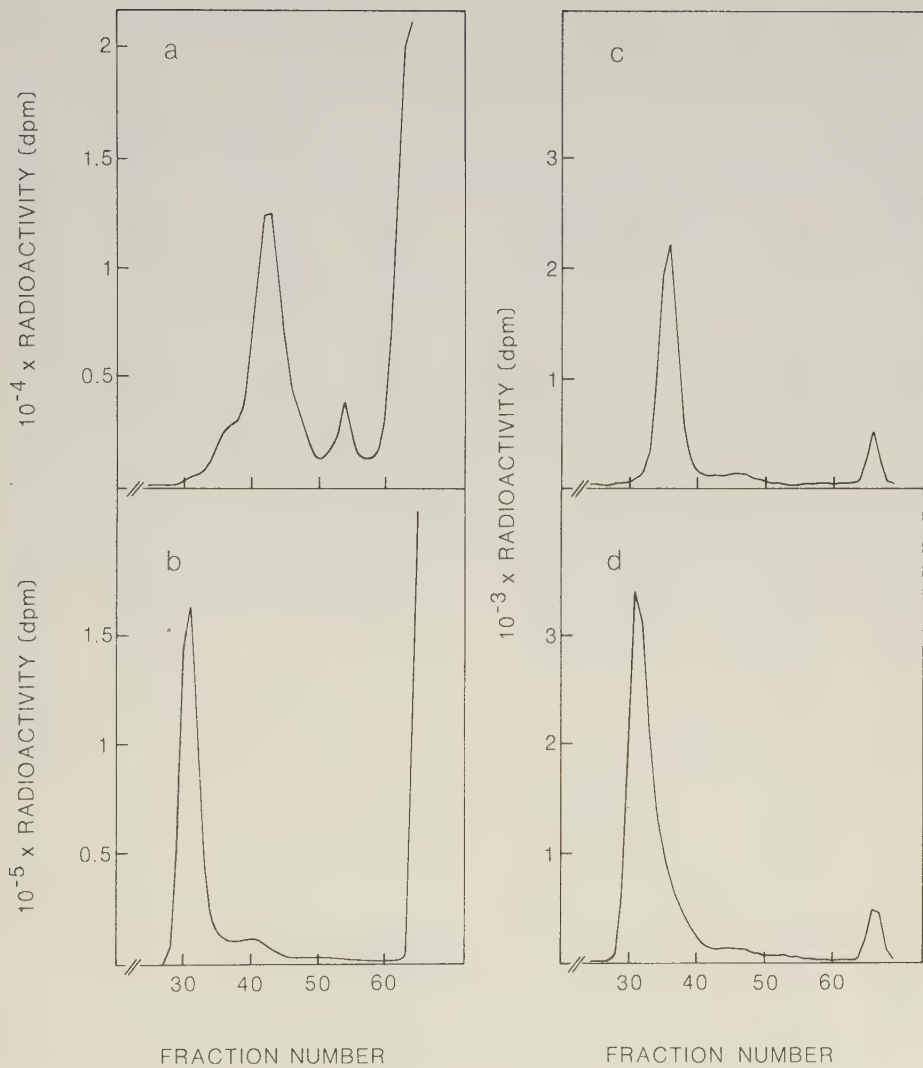


Figure 2.

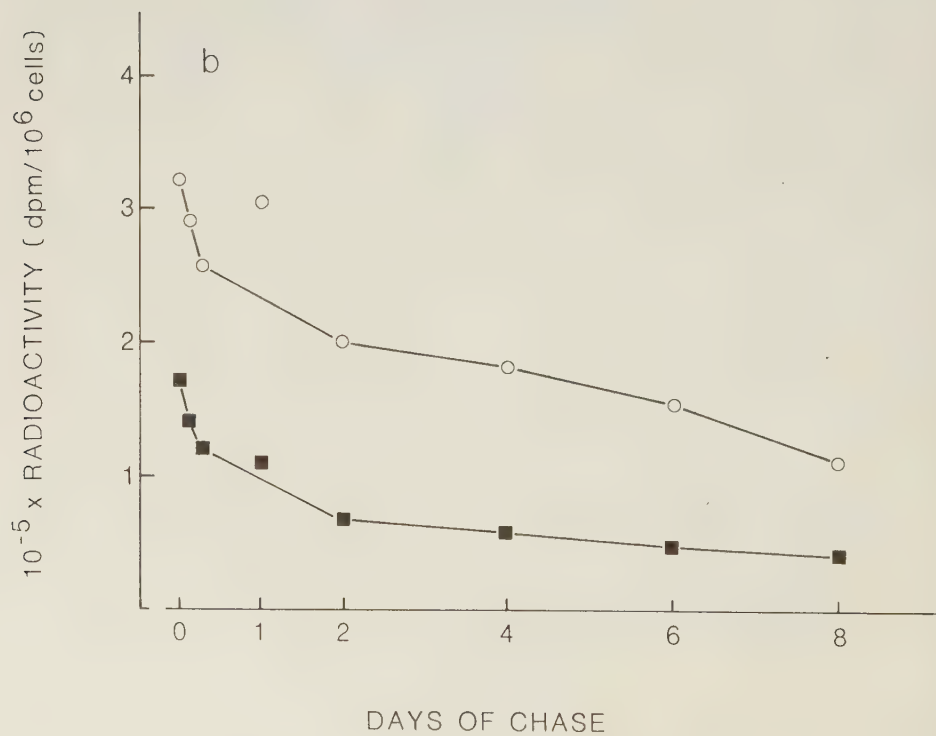
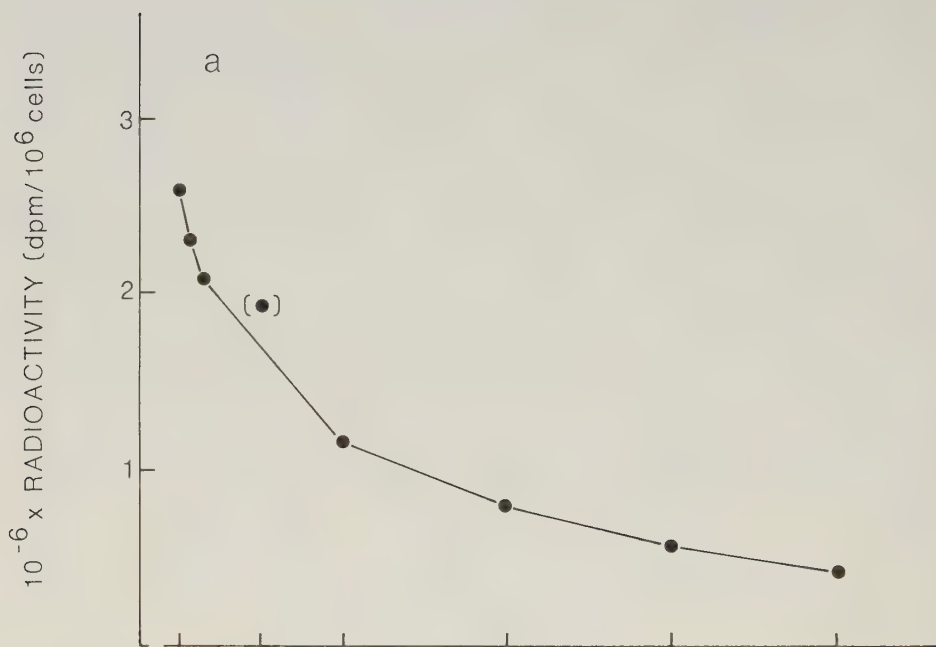


Figure 3.

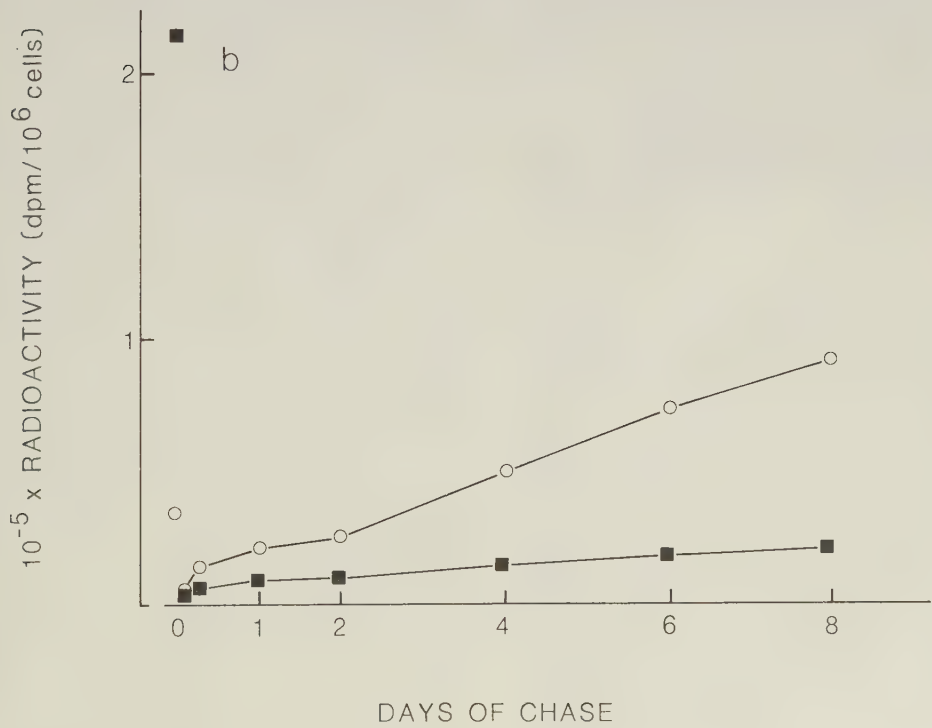
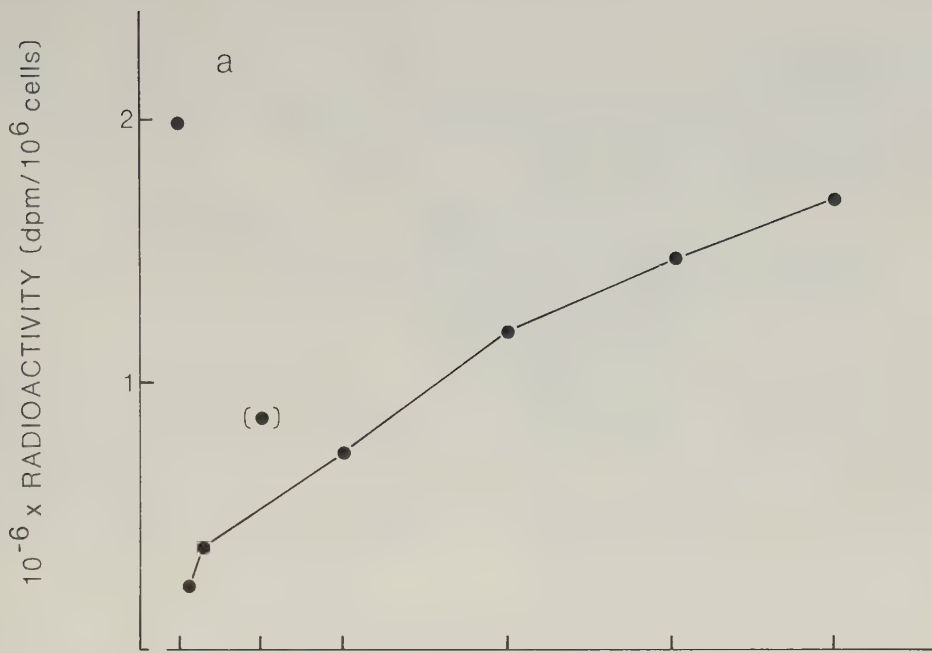


Figure 4.

Two Novel Matrix Proteins Isolated from Articular Cartilage Show Wide Distributions among Connective Tissues*

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Two proteins of $M_r = 58,000$ and $59,000$, respectively, were purified from 4 M guanidinium chloride extracts of articular cartilage by dissociative CsCl-density gradient centrifugation followed by gel chromatography on Sephadex G-200 and ion exchange chromatography on DEAE-cellulose. The two proteins differ in ionic properties and only the one with $M_r = 59,000$ bound to the ion exchanger. Although the two proteins showed dissimilar peptide patterns after proteolysis, their amino acid composition was similar, with very high contents of leucine and aspartic acid/asparagine.

The two proteins showed no cross-reactivity in radioimmunoassays. By use of these assays, the proteins were demonstrated in extracts of most connective tissues, with high contents of about 0.1% of tissue wet weight determined in several types of cartilage. Among the non-cartilage connective tissues, tendon and sclera had the highest contents of the proteins, i.e. about 0.1% of the tissue wet weight. Bone extracts, on the other hand, contained insignificant amounts of the proteins. Only the $M_r = 59,000$ protein was detected in serum, its concentration being about $33 \mu\text{g/l}$.

Both proteins were shown to be localized in the extracellular matrix of cartilage, predominantly in the territorial matrix, by using indirect immunofluorescence.

In many studies of cartilage, much interest has been focused on proteoglycans and collagens being the major tissue constituents. However, almost 2 decades ago, Szirmai *et al.* (1) pointed out that the sum of the protein content of proteoglycans and collagen constitutes less than 50% of the total protein in nasal cartilage. Subsequently, several novel proteins have been isolated from various cartilages (for references, see Ref. 2). Interestingly, though, only relatively few major proteins can be detected by SDS¹-polyacrylamide gel electrophoresis of cartilage extracts (3). Examples are the link proteins, the 148-kDa protein, the 36-kDa protein, and a large multimeric protein (4). Among these, the 148-kDa protein is particularly prominent in adult tracheal cartilage, where it constitutes some 5% of the tissue wet weight (5). Notably, however, this protein is not present in articular cartilage (3),

which on the other hand, contains another very large prominent multimeric protein (4). In addition, articular cartilage contains a major protein component that migrates upon SDS-polyacrylamide gel electrophoresis to a position similar to that of the reduced subunit of the 148-kDa protein (3). These articular cartilage proteins in contrast contain a single polypeptide chain as their mobility is not affected by reduction.

The present paper describes the isolation and characterization of these two proteins from articular cartilage and also show their wide distribution within connective tissues.

MATERIALS AND METHODS

Bovine articular cartilage was prepared from the fetlock joints of steers approximately 2 years old and sliced into fine pieces of less than 1-mm cross-section. Extraction was done with 15 volumes of 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, also containing 10 mM EDTA, 5 mM benzamidine chloride, 0.1 M 6-aminoheptanoic acid, and 5 mM *N*-ethylmaleimide for 24 h at 3 °C as previously described (6).

All urea solutions were made fresh from stock passed through a mixed bed ion exchange column immediately before use.

Separation of Proteins and Proteoglycans—The extract was adjusted to a density of 1.35 g/ml by the addition of solid CsCl. Proteoglycans were separated from proteins using equilibrium centrifugation in a self-forming CsCl-gradient. A Beckman 42.1 angle rotor equipped with 38.5-ml Quick-seal tubes was used and centrifugation was done at 33,000 rpm ($83,900 \times g$, $r_{\text{av}} = 6.88$ cm) for 70 h at 16 °C. The gradient was sectioned in four equal portions with the aid of a Beckman tube slicer.

Gel Chromatography—The two uppermost fractions of the CsCl-gradient (D3 and D4) were pooled, concentrated, and transferred by diaflow into 4 M guanidinium chloride, 5 mM sodium phosphate, pH 7.4, by ultrafiltration over an Amicon PM-10 membrane. The final volume was 15 ml. This concentrate was chromatographed on a Sephadex G-200 column (3×148 cm, Pharmacia P-L Biochemicals), eluted at 20 °C with 4 M guanidinium chloride, 5 mM sodium phosphate, pH 7.4.

DEAE-cellulose Chromatography—The pooled fraction from the Sephadex G-200 chromatography was dialyzed extensively against water and lyophilized. This material was dissolved in 10 ml of 7 M urea, 10 mM Tris/HCl, pH 7.0, and chromatographed on a column (1.6×15 cm) of DEAE-cellulose (DE52, Whatman), equilibrated with the same urea solution. After nonbound material had been eluted with the urea solution, bound material was eluted at 14 °C with a gradient of 0–0.5 M NaCl in the urea solution. Effluent fractions were monitored for protein contents by absorbance at 280 nm.

Gel Chromatography on Superose 6—A column (0.65×145 cm) of Superose 6 (Pharmacia P-L Biochemicals), eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, was used to check the final purity of the pooled fractions from the DEAE-cellulose chromatography. Samples (0.25 ml; 4 mg/ml) were dissolved in the guanidinium chloride solution and chromatographed at 20 °C. The column effluent was continuously monitored for absorbance at 280 nm using a Uvicord S monitor (LKB).

SDS-Polyacrylamide Gel Electrophoresis—Proteins in samples of fractions to be analyzed, were precipitated with 10 volumes of ethanol containing 0.05 M sodium acetate and recovered by centrifugation as described elsewhere (7). Samples containing guanidinium chloride

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¹ The abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; l, liter; PBS, phosphate-buffered saline.

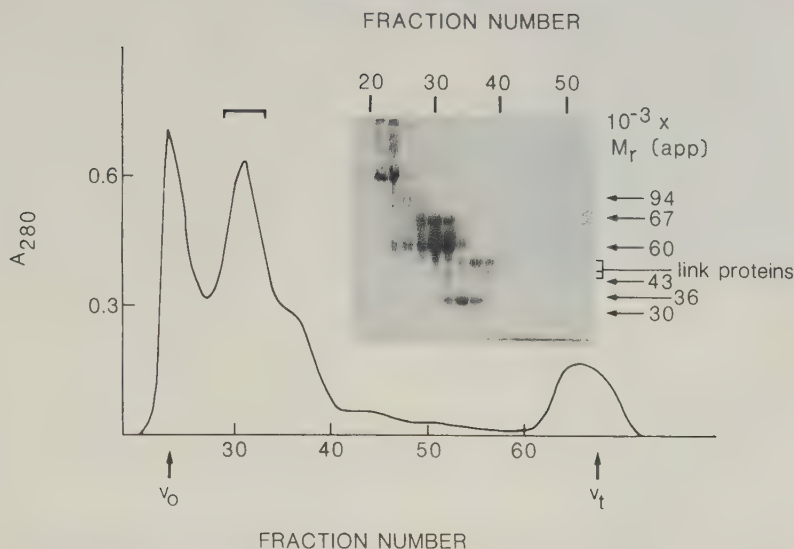


FIG. 1. Sephadex G-200 chromatography of the top fractions (buoyant density less than 1.34 g/ml) from a dissociative CsCl-density gradient centrifugation of an articular cartilage extract. The column (3×148 cm) was eluted with 4 M guanidinium chloride, 5 mM sodium phosphate, pH 7.4. Proteins in samples of every second fraction were electrophoresed under reducing conditions on 8% SDS-polyacrylamide gels (inset). The fractions indicated by a bar were pooled. V_0 and V_t indicate the void and total volumes of the column, respectively. The migration positions of the link proteins are indicated.

ere reprecipitated to remove traces of guanidine. The precipitates are dissolved in sample buffer and electrophoresed on 8% SDS-polyacrylamide gels using the buffer system of Neville as described elsewhere (8). Ferguson plots (9) were constructed after electrophoresis on 6, 7, 8, 9, and 10% (w/v) polyacrylamide gels containing 2.6% (v/v) bisacrylamide as a cross-linker of samples reduced with 1% mercaptoethanol.

Peptide Mapping—Proteins were [125 I]iodine-labeled, using the chloramine-T procedure, digested with trypsin, and analyzed by two-dimensional high voltage electrophoresis and thin layer chromatography on silica plates as described elsewhere (10).

Radioimmunoassay—Samples (200 μ g) of each of the two proteins are dissolved in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4, mixed with Freund's complete adjuvant, and injected subcutaneously several sites in the neck of rabbits. Booster doses were given after weeks with the antigens (100 μ g) mixed with Freund's incomplete adjuvant. The rabbits were bled by venesection in the ear.

Radioimmunoassay of the two proteins was done essentially as described elsewhere (3). The antigen dissolved in 7 M urea was labeled with [125 I]iodine using the chloramine-T procedure as described (3, 1). Maximal precipitation with antibodies was 80–85% for both proteins. For assay, the labeled antigen (50,000 dpm) was dissolved in 50 μ l of 0.4% SDS, 0.1% bovine serum albumin (BSA), 10 mM sodium phosphate, pH 7.4, and mixed with an equal volume of sample the same SDS/BSA solution. Twice the volume (200 μ l) of antibody eluted in 2% Triton X-100, 0.25% BSA, 0.45% NaCl, 0.05% Na₂S₂O₅, 5 mM sodium phosphate, pH 7.4, was added to achieve a final antibody dilution of 1:1600. After incubation overnight at 4 °C, a cond antibody (100 μ l), i.e. sheep anti-rabbit IgG, at an appropriate dilution in 5% polyethylene glycol 6000, 5 mM sodium phosphate, pH 7.4, was added. After another overnight incubation at 4 °C, 2.5 volumes of 0.5% BSA, 0.9% NaCl, 0.01% Na₂S₂O₅, 50 mM sodium phosphate, pH 7.4, was added and the precipitate was recovered by centrifugation. Radioactivity was counted in a γ counter, and quantities were calculated by use of a spline function.

The specificity of the radioimmunoassay was checked by chromatography an extract of bovine articular cartilage on the Superose 6 column eluted with 4 M guanidinium chloride. Samples of the fractions were precipitated with ethanol, redissolved in SDS, and taken

to radioimmunoassay as described above.

Contents of the antigens in extracts of a number of bovine tissues were determined. Tissue pieces were powdered in liquid N₂ as described elsewhere (5) and extracted for 24 h at 4 °C with 15 volumes of 4 M guanidinium chloride, containing the protease inhibitors listed above. Proteins and proteoglycans were precipitated with ethanol (7). The precipitates were dried and dissolved in 0.4% SDS containing 0.1% BSA, 10 mM sodium phosphate, pH 7.4. Radioimmunoassay was done as described. Contents of the antigens were expressed on a wet weight basis.

Chemical Methods—Contents of amino acids were determined after hydrolysis of samples in 6 M HCl (Aristar, British Drug House, Poole, Dorset, England) under argon for 24 h at 110 °C. Contents of hexosamines were determined after hydrolysis in 4 M HCl for 10 h at 100 °C under argon, both analyses done with a Durrum amino acid analyzer. Contents of neutral sugars were determined after hydrolysis of samples in 2 M trifluoroacetic acid for 3 h at 100 °C. Samples were passed through a mixed bed ion exchange resin and neutral sugars were separated using the Bio-Rad HPX-87P carbohydrate analysis column eluted with water. Contents of monosaccharides in the column effluent was monitored using the orcinol reaction adapted for the Technicon Auto-Analyzer (12).

Immunolocalization—A 5-mm punch biopsy of fetlock joint articular cartilage from a 2-year-old steer was frozen in liquid N₂. The specimen was mounted on a cryostat-stage and sectioned in 4- μ m-thick sections using a cryostat. The sections were taken up on glass plates which had been previously coated with a solution containing gelatin (0.5%, w/v) and potassium chromium sulfate (0.05% w/v). Before immunostaining, the sections were fixed in absolute ethanol, left to dry, and then digested with testicular hyaluronidase in 0.15 M sodium chloride, 5 mM phosphate, pH 7.4 (Sigma, type 15, 1 mg/ml) by adding a 20- μ l drop to each section. After incubation for 1 h at 37 °C in a humidified chamber, the sections were rinsed with 0.15 M NaCl, 5 mM sodium phosphate, 0.05% (w/v) Tween, pH 7.4 (PBS-Tween). To block nonspecific binding of rabbit immunoglobulins, each section was incubated for 30 min at room temperature with a 20- μ l drop of fetal calf serum (diluted 1:10 with PBS-Tween). Incubation with first antibody (20 μ l, diluted 1:10 in PBS-Tween) was for 30 min at room temperature. Negative controls were similarly incu-

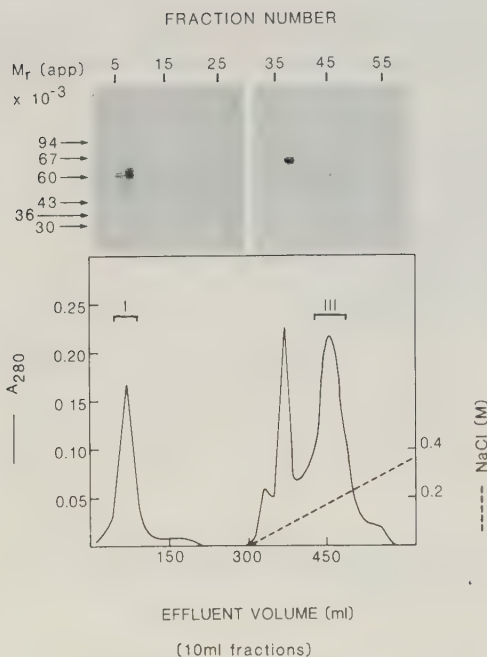


FIG. 2. DEAE-cellulose chromatography of the pool from the Sephadex G-200 chromatography. A column (1.6×15 cm) of DE52 was eluted with 7 M urea, 10 mM Tris/HCl, pH 7.0, followed by a linear gradient of 0–0.5 M NaCl in the urea solution. Proteins in samples of every second fraction were electrophoresed under reducing conditions on 8% SDS-polyacrylamide gels (*inset*). The fractions indicated by bars were pooled.

bated with first antibody that had been preincubated overnight with the antigen (100 μ l, diluted 1:10 with PBS-Tween and mixed with 10 μ g of antigen). After rinsing with PBS-Tween, incubation with second antibody (swine anti-rabbit IgG, fluorescein isothiocyanate conjugate, Orion Diagnostica, diluted 1:10 in PBS-Tween), was done for 30 min at room temperature. After rinsing with PBS-Tween, slides were mounted with coverslips in glycerol/water (9:1) before microscopy.

RESULTS

Purification—Bovine articular cartilage was extracted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, containing protease inhibitors, and the extract was directly subjected to CsCl density gradient centrifugation. The major portion of material showing absorbance at 280 nm was recovered in the top half of the CsCl-gradient (fraction D3 + D4, data not shown). These fractions were concentrated, transferred into 4 M guanidinium chloride by ultrafiltration, and chromatographed on Sephadex G-200 eluted with 4 M guanidinium chloride (Fig. 1). The proteins present in every second fraction were taken to SDS-polyacrylamide gel electrophoresis (Fig. 1, *inset*). The void volume peak contained some collagen and material too large to enter the gel. In a major peak of included material, components with apparent $M_r = \sim 60,000$ appeared. A protein with an apparent $M_r = \sim 35,000$ eluted partly resolved at the end of the peak. The trailing shoulder contained the link proteins as indicated. The fractions containing the proteins with apparent $M_r = \sim 60,000$ were pooled as indicated, dialyzed against water, and lyophilized.

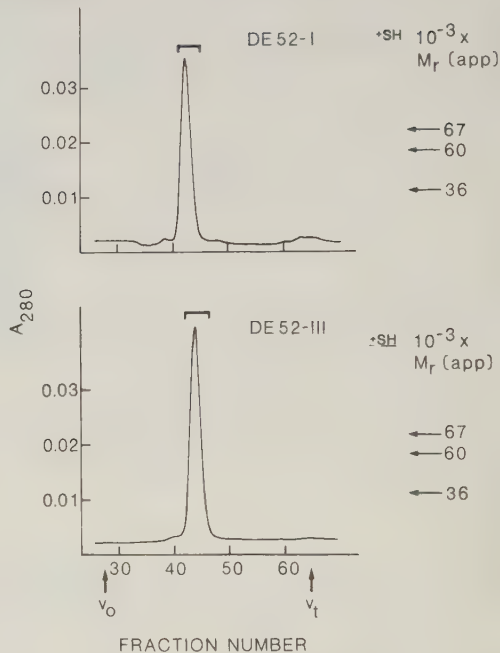


FIG. 3. Superose 6 chromatography of the two pools recovered from the DEAE-cellulose chromatography. A Superose 6 column (0.65×147 cm) was eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. Samples (1 mg) of the two components recovered from the DEAE-cellulose chromatography were dissolved in 250 μ l of 4 M guanidinium chloride and applied to the column. The effluent was continuously monitored for absorbance at 280 nm, DE52-I (top) pool I ($M_r = 58,000$ protein) and DE52-III (bottom) pool III ($M_r = 59,000$ protein). The fractions indicated were pooled, and the materials recovered after dialysis were electrophoresed on 8% SDS-polyacrylamide gels (*inset*). V_0 and V_t indicate the void and total volumes of the column, respectively.

lized. The material (60 mg) was recovered from 10 g of fresh cartilage) was chromatographed on DEAE-cellulose (Fig. 2). Samples of every second fraction were electrophoresed on SDS-polyacrylamide gels (Fig. 2, *inset*).

The material not bound to the column consisted essentially only of a protein with an apparent $M_r = \sim 60,000$. The late eluting peak contained another major protein, with an apparent M_r of close to 60,000. The intermediate peak contained a component at the position of albumin, which also showed the albumin typical slight shift in mobility upon reduction. Fractions (I and III) were pooled as indicated, dialyzed against water, and lyophilized. The yield in pool I was 11.2 mg and in pool III 17.5 mg from 10 g of fresh cartilage. To check the purity of these two preparations, they were chromatographed on a high resolution Superose 6 column, eluted with 4 M guanidinium chloride (Fig. 3). They showed very similar elution properties, although the one with a somewhat lower apparent molecular mass by SDS-polyacrylamide gel electrophoresis surprisingly eluted in a somewhat less retarded fashion. The gel chromatography nevertheless indicated purity of the two proteins. They did not alter their electrophoretic mobility upon reduction, indicating a single chain non-disulfide bonded structure.

Chemical Characterization—Both proteins were shown to

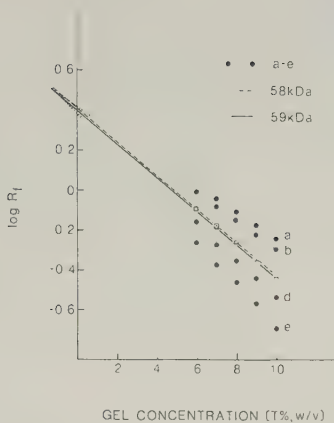


FIG. 4. Ferguson plots of the purified proteins. Samples of the purified proteins were electrophoresed under reducing conditions on SDS-polyacrylamide gels having the indicated concentrations of polyacrylamide. The graph for reference protein (c) (catalase subunit, beef liver, $M_r = 60,000$) overlaps that of the two sample proteins and is not shown. Reference proteins were: a, lactate dehydrogenase subunit, beef heart ($M_r = 36,000$); b, ovalbumin, egg white ($M_r = 43,000$); d, albumin, bovine serum ($M_r = 67,000$); e, phosphorylase b, rabbit muscle ($M_r = 94,000$).

TABLE I
Composition of the two proteins

Amino acid	58-kDa	59-kDa
	residues/100	
Aspartic acid	120	144
Threonine	32	33
Serine	73	88
Glutamic acid	86	119
Proline	117	80
Glycine	37	49
Alanine	37	44
Cysteine	13	9
Valine	48	46
Methionine	7	3
Isoleucine	53	39
Leucine	142	139
Tyrosine	32	54
Phenylalanine	49	37
Histidine	25	29
Lysine	53	35
Arginine	75	53
	$\mu\text{g}/\text{mg}$	
GlcN	25.5	27.0
GalN	17.0	10.8
Galactose	9.9	7.9
Glucose	3.1	5.1
Mannose	16.8	12.2
Xylose	1.4	1.1
Fucose	9.4	8.8
Sialic acid	3.3	2.0
Sum carbohydrates	86.4	63.9

behave ideally upon SDS-polyacrylamide gel electrophoresis of reduced samples, since Ferguson plots (9) converged toward the same point as that of reference proteins. Therefore, their electrophoretic mobilities can be used to calculate molecular masses, which were found to be 58,000 (I) and 59,000 (III), respectively (Fig. 4).

The composition of the two proteins was determined (Table I). Their amino acid composition differed somewhat, mainly

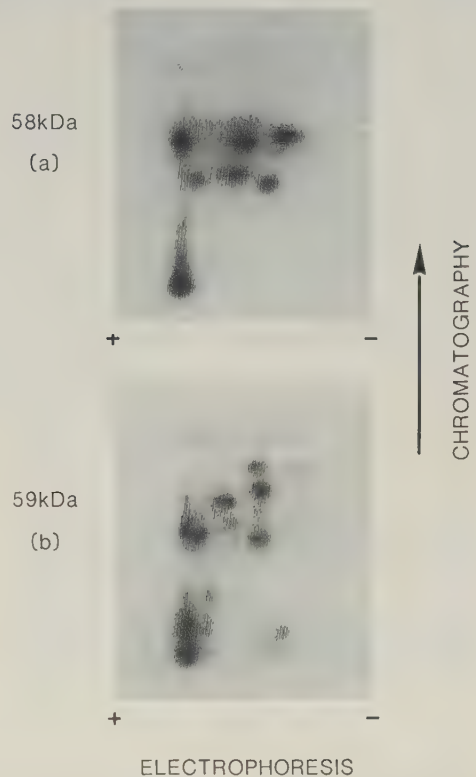


FIG. 5. Tryptic peptide patterns of the two proteins. Samples of the 58-kDa (a) and the 59-kDa (b) proteins were labeled with $[^{125}\text{I}]$ iodine, digested with trypsin, and separated in two dimensions on thin layer plates of silica as described elsewhere (10).

with respect to contents of glutamic acid/glutamine and tyrosine. The proline content of the 58-kDa protein was unusually high. Both proteins, however, had very high contents of leucine and of aspartic acid/asparagine as previously found for other connective tissue proteins, including the small proteoglycans (10). Interestingly, the galactosamine content of both proteins was high. Since no galactosamine was converted to galactosaminitol by alkaline borohydride treatment, it does not appear that these galactosamine residues participate in *O*-glycosidic linkage to protein. A possible candidate for providing the galactosamine would be chondroitin sulfate/dermatan sulfate. No glycosaminoglycan chains of M_r exceeding 4000 could be detected, however (data not shown). Thus, the proteins do not appear to contain the commonly found type of rather large chondroitin sulfate/dermatan sulfate chains. Furthermore, neither protein reacts with antibodies raised against large (PG-L) and small (PG-Sm1 and -2) interstitial proteoglycans, respectively (data not shown). Since *N*-glycosidically linked oligosaccharides are not known to contain galactosamine residues, it is possible that these proteins contain oligosaccharides of a novel type.

The non-identity of the two proteins was shown by peptide mapping after trypsin digestion (Fig. 5). The patterns are very different, showing that the proteins presumably repre-

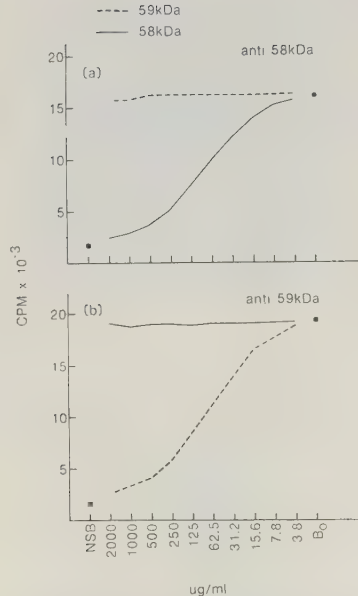


FIG. 6. Radioimmunoassay of the 58- (a) and 59-kDa (b) proteins, respectively. For experimental details, see "Materials and Methods." Note the complete absence of cross-reaction between the two proteins.

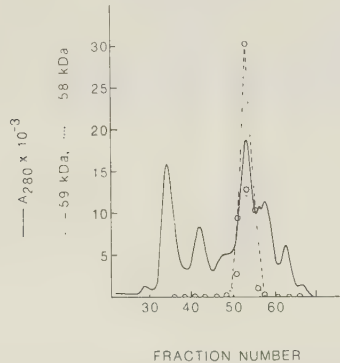


FIG. 7. Gel chromatography of an extract of articular cartilage assayed with radioimmunoassays for the 58- and 59-kDa cartilage proteins. A 4 M guanidinium chloride extract of articular cartilage was chromatographed on serially coupled columns of Sephacryl S-500 (0.65 × 47 cm) and Superose 6 (0.65 × 147 cm) eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. The effluent was continuously monitored for absorbance at 280 nm (—). The 58- (---) and 59- (---) kDa proteins quantified by radioimmunoassay of each fraction.

sent different gene products, although their overall amino acid compositions are similar.

Immunoassay and Distribution among Tissues—In order to allow studies of the presence of the proteins in various tissues, antibodies were raised in rabbits, and radioimmunoassays were developed (Fig. 6). Since extracts of connective tissues contain large amounts of material that is not soluble in

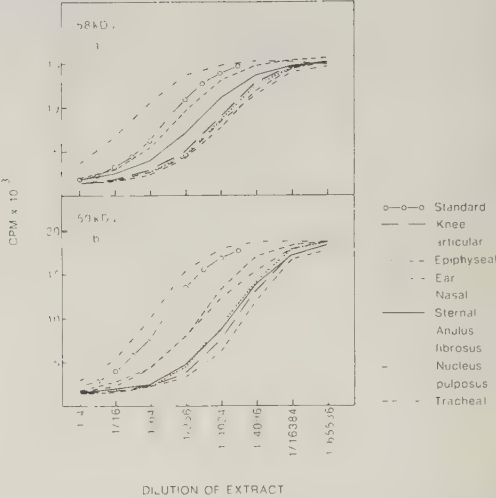


FIG. 8. Presence of the 58-kDa and the 59-kDa proteins in various bovine cartilages. Samples of 4 M guanidinium chloride (20 ml/g) extracts of bovine knee articular, nasal, tracheal, ear, epiphyseal, and sternal cartilages as well as the anulus fibrosus and the nucleus pulposus of the intervertebral disc were precipitated with ethanol. The precipitates were dissolved in aqueous SDS, and serial dilutions were used as inhibitors in the radioimmunoassays for the 58- (a) and 59- (b) kDa proteins, respectively. Dilution factors refer to the dilution of the original extract.

TABLE II		
Contents of 58-kDa and 59-kDa proteins in different cartilages		
	58-kDa	59-kDa
mg/g wet wt tissues		
Tracheal	1.29	3.00
Knee articular	0.86	1.98
Epiphyseal	0.18	1.70
Sternal	0.39	1.46
Anulus fibrosus	0.76	1.38
Nasal	0.98	1.34
Ear	1.04	0.41
Nucleus pulposus	0.03	0.07

nondenaturing buffers, we preferred to use a procedure where the antigen is dissolved in SDS. Excess SDS is then bound in mixed micelles with nonionic detergent (3). Under these conditions, sufficient SDS is retained by the proteins to keep them in solution. The two proteins showed no cross-reactivity in these assays in further support of their different nature (Fig. 6).

The specificity of the assays was tested by analysis of the proteins present in an extract of articular cartilage which had been subfractionated by chromatography on two serially coupled columns of Sephacryl S-500 and Superose 6 (Fig. 7). Radioimmunoassay was used to determine contents of the two proteins in the fractions. Antigenic material was only detected in one peak, corresponding to the elution position of the 58- and 59-kDa proteins, demonstrating the specificity of the assays.

The presence of the proteins in a number of tissues was tested, using ethanol precipitates of guanidinium chloride extracts as antigens in the radioimmunoassays. Both proteins were found to be present in variable amounts in a number of bovine cartilages, including intervertebral disc (Fig. 8 and

FIG. 9. Demonstration of the 58-kDa and the 59-kDa proteins in various bovine tissues by radioimmunoassay. Proteins in 4 M guanidinium chloride extracts (20 ml/g wet tissue) of various bovine tissues were precipitated with ethanol and dissolved in SDS, and serial dilutions were used as inhibitors in the radioimmunoassays for the 58-kDa protein (a, c) and the 59-kDa protein (b, d). Dilution factors refer to the dilution of the original extract.

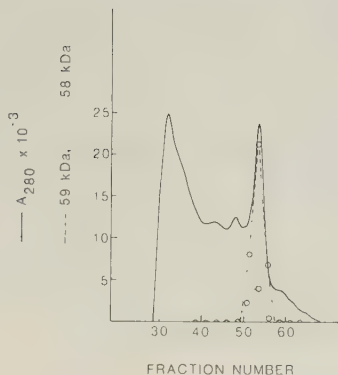
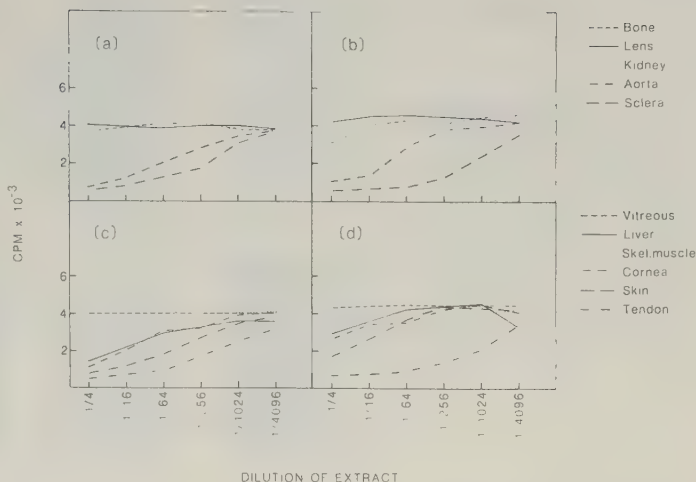


FIG. 10. Molecular sieve chromatography of the antigens present in tendon extracts. A 4 M guanidinium chloride extract (20 ml/g) of tendon was chromatographed on serially coupled columns of Sephacryl S-500 (0.65 × 47 cm) and Superose 6 (0.65 × 147 cm) eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. The effluent was continuously monitored for absorbance at 280 nm (—). Proteins in samples of the fractions were precipitated with ethanol and used as inhibitors in the radioimmunoassays for the 58 kDa (·····) and 59 kDa (---) proteins.

Table II). The cartilages had different contents of the proteins, ranging from less than 0.1% to 0.3% of tissue wet weight, tracheal cartilage containing more than any of the others. As expected, articular cartilage also contained relatively large amounts of both proteins. Interestingly, nucleus pulposus contained much less of the proteins than any of the cartilages proper. Furthermore, most other connective tissues also contain components that reacted in the assay, with the slopes of the inhibition curves being similar between the tissues (Fig. 9). Sclera and tendon appeared to contain larger amounts of the proteins than the other non-cartilage tissues. To further identify the reactive components, a sample of the extract from tendon was chromatographed on the serially coupled Sephacryl S-500-Superose 6 columns. Only material chromatographing at the position of the 58-kDa and 59-kDa proteins was found to inhibit in the radioimmunoassays, showing that the

reactivity represents the proteins (Fig. 10). The contents of the proteins in tendon and sclera were of the same magnitude as that in an average cartilage. Interestingly, serum appeared to contain material reacting only in the assay for the 59-kDa protein, where two separate dilutions of serum gave similar values of 33 $\mu\text{g/l}$, i.e. the protein in serum is identical with the isolated 59-kDa protein. No inhibition was obtained with the 58-kDa protein, showing that this protein was not present in the circulation.

Immunolocalization—The distribution of the two proteins in articular cartilage was studied by using the indirect immunofluorescence technique. With both antisera, a strong matrix staining was obtained, showing the extracellular location of the proteins (Fig. 11). The staining was blocked by preincubation of the antiserum with the respective protein, showing the procedure specificity. Interestingly, the most intense staining was observed in the territorial matrix, possibly indicating a preferential location of both proteins to this compartment. Alternatively, the differences in staining intensities may be due to variations in the accessibility of epitopes in different parts of the tissue matrix.

DISCUSSION

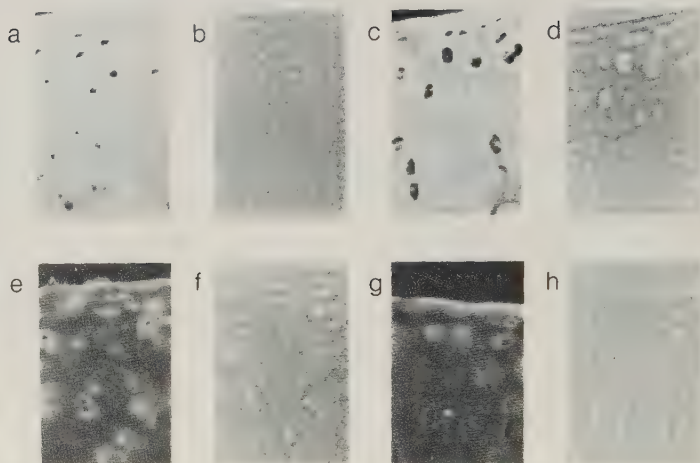
The compositions of the 58- and 59-kDa proteins, with high contents of a few typical amino acids such as leucine and aspartic acid, are similar to those of some other connective tissue macromolecules, i.e. the small proteoglycans (10) and a 36-kDa protein (2).² The elution behavior on DEAE-cellulose indicates that the 59-kDa protein is anionic, possibly due to the high contents of glutamic acid and aspartic acid. Both proteins are made up of a single polypeptide chain each and appear to contain no or few disulfide bridges, since their mobilities on SDS-polyacrylamide electrophoresis are not affected by reduction.

The two proteins represent distinct molecules, i.e. different gene products, giving completely different peptide patterns and not sharing any antigenic structures.

Unlike the trimeric 148-kDa protein previously isolated from tracheal cartilage, the 58- and 59-kDa proteins isolated from articular cartilage are present in all cartilages studied.

² D. Heinegård, T. Larsson, Y. Sommarin, A. Franzén, M. Paulsson, and E. Hedbom, unpublished results.

FIG. 11. Immunolocalization of the 58-kDa and the 59-kDa proteins. Sections of articular cartilage were digested with testicular hyaluronidase and incubated with antibodies to the 58-kDa protein (a, e) and the 59-kDa protein (c, g). For control (e, g), the antibody reactivity was blocked by preincubation with the respective antigen. Bound immunoglobulins were detected by the use of fluorescein isothiocyanate labelled swine-anti-rabbit IgG. Phase contrast pictures of the respective section is also shown (b, d, f, h).



The two proteins are presumably also expressed by a number of different cell types, since they were shown to be present in many different connective tissue matrices. They were, however, not present in tissues like lens and the vitreous body. Liver, skeletal muscle, and bone showed very weak reactivity, possibly nonspecific. Differences in the proportion of the two proteins between tissues were observed, for example in kidney, where only the 58-kDa protein was detected and in bone where only the 59-kDa protein was detected. Serum contained only the 59-kDa protein, probably released from the connective tissues.

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Binding of isolated chondrocytes to
extracellular matrix proteins from
cartilage

by

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SYNOPSIS

Demonstration of cell attachment was used to detect interactions between chondrocytes and extracellular matrix proteins/proteoglycans isolated from cartilage. Chondrocytes which had been maintained in spinner culture attached to plastic surfaces coated with either fibronectin, osteopontin (from bone), collagenII, the 36kDa- or the 58kDa-proteins isolated from cartilage. Attachment to fibronectin and osteopontin was inhibited by competition with an Arg-Gly-Asp containing peptide, specific for the cell binding region of fibronectin, whereas attachment to the 36kDa- and the 58kDa-proteins was not affected indicating an alternative mechanism for attachment. The 59kDa- and the 400kDa-proteins from cartilage gave a variable, low degree of attachment.

Freshly isolated chondrocytes showed a slightly more pronounced attachment to proteoglycans and to the 59kDa-protein than cells maintained in spinner culture, while attachment to the other matrix constituents was the same.

Abbreviations: BSA - bovine serum albumin, FN - fibronectin, OPN - osteopontin, CII - collagen II, HA - hyaluronic acid, PG-LA - large aggregating proteoglycan, PG-S - small proteoglycan.

INTRODUCTION

The cells in cartilage are dispersed in an abundant extracellular matrix. To maintain the functional integrity of the tissue the cells must be able to monitor the composition of their surrounding matrix. The mechanism for this is not known, but experiments with cultured cells have shown that addition of low concentrations of hyaluronic acid to the culture medium will result in lower synthesis of proteoglycans (Wiebkin & Muir, 1973, 1977; Handley & Lowther, 1976).

Addition of high concentrations of proteoglycan to chondrocytes on the other hand has an opposite effect in that it reportedly stimulates proteoglycan synthesis (Nevo & Dorfman, 1972; Schwartz & Dorfman 1975; Handley & Lowther, 1977). Experiments of this nature indicate that the monitoring of extra cellular matrix composition may be mediated via interaction with receptors or structures at the cell surface. In support proteoglycans added to cultured cells can bind to hyaluronic acid at the chondrocyte cell surface (Sommarin & Heinegård, 1983). Similarly interactions with collagen II has been shown and a receptor has been isolated from chondrocytes (Mollenhauer & von der Mark, 1983). A plasmaprotein with the capacity to promote attachment of chondrocytes to collagen has also been described (Hewitt et al., 1980, 1982).

We have isolated and characterized several non collagenous proteins from cartilage a 148kDa-protein (Paulsson & Heinegård, 1981), a 58kDa-protein, a 59kDa-protein (Heinegård et al., 1986), and a 36kDa-protein (to be published). Their functions are not known. The aim of the present investigation is to reveal if any of these proteins have the potential to interact with structures at the chondrocyte cell surface and relay information to the cells for composition and properties of the matrix. Since some of the isolated proteins are not soluble in physiological buffers we have chosen to study binding of chondrocytes to plastic surfaces coated with the different proteins.

EXPERIMENTAL

Tissue culture media was Ham's F12 from Flow Laboratories. Collagenase was from Worthington Biomedical Corp. (Freehold, N.J., USA). Bovine serum albumin (BSA), Cohn fraction V was from Sigma Chemicals (St. Louis, Mo). Fibronectin was isolated from bovine plasma by gelatin- and heparin-Sepharose affinity chromatography (Yamada, 1984). CollagenII was isolated from bovine tracheal cartilage by pepsin digestion, precipitation with sodium chloride and CM-cellulose chromatography (Miller, 1972). The 58kDa-, 59kDa- and 148kDa protein from bovine cartilage were isolated as described (Heinegård et al, 1986 and Paulsson & Heinegård, 1981, respectively). The 36kDa protein was from bovine tracheal cartilage and isolated by a sequence of CsCl-density gradient centrifugation, DEAE- and CM-cellulose chromatography (to be published). A protein of more than 400 kDa molecular weight was isolated from articular cartilage and may be the same as a protein studied by Spitz Fife and Brandt, 1984. Bovine osteopontin was a preparation described elsewhere (Franzén & Heinegård, 1985). Dr. E. Ruoslahti kindly provided the hexapeptides.

Isolation of chondrocytes from tracheal cartilage

Tracheal cartilage from 4-6 months old calves were dissected free of perichondrium and cut into thin slices. Cells were liberated by collagenase digestion at 37°C for 8 hours in 0.2% collagenase in Earles basal salt solution supplemented with two times the amount of amino acids in Eagle's minimum essential medium and 50 units each of penicillin and streptomycin sulphate/ml. The cells were transferred to Ham's F12 medium buffered with 20 mM HEPES and supplemented with 1 mg BSA/ml and antibiotics as above (this medium is referred to as F12HB). They were either used directly for experiments or transferred to spinner culture in Ham's F12 medium supplemented as above with either 1 mg BSA/ml or 20 µg dextran sulphate/ml. Cells were kept in spinner culture for 24-48 hours before being transferred to F12HB. For use in experiments the cells were diluted to 0.5×10^6 /ml.

Preparation of other cell types.

Chondrocytes from Swarm rat chondrosarcoma were isolated essentially as above but digested with collagenase for only 2 hours. Rat hepatocytes prepared by collagenase perfusion, a gift from K. Musil, were washed and transferred to F12HB before experiment. Cultured bovine scleral fibroblasts, a gift from

Dr.A.Malmström, were briefly trypsinized, rapidly washed three times in Ham's F12 medium with soy bean trypsin inhibitor (0.5 mg/ml) and transferred to F12HB medium.

Coating of plastic surfaces

For coating, solutions with 5 ug/ml of BSA, collagen II, 36kDa, 58kDa, 59kDa, 148kDa, 400kDa or osteopontin in 4 M guanidinium chloride, 50 mM Tris-HCl pH 7.6 were used. Large aggregating proteoglycan (PG-LA) at 100ug/ml or small nasal cartilage proteoglycan at 10 ug/ml were used for coating. Commercially available hyaluronic acid (Sigma), that contains some protein, was coated at 10 ug/ml in phosphate buffered saline (PBS). Fibronectin were coated at 5 ug/ml in 0.1 M NaHCO₃. In all cases 100 ul of coating solution were added to each well of Nunc (Roskilde, Danmark) 96 well polystyrene immunoplates (cat no 4-39454). After coating overnight at room temperature the wells were rinsed with PBS and aftercoated with 100 ul of 1 mg BSA/ml PBS for 2 hours. The wells were then rinsed with PBS and used directly for binding experiments. With these coating procedures highly reproducible results are obtained in ELISA for the different macromolecules even when only 1/10 of the concentration is used.

Attachment experiments

100 ul of cell suspension (50 000 cells) was added to each well and the cells were allowed to attach for 1-2 hours at 37°C. Cells not attached were removed by careful rinsing, three times with PBS. The number of attached cells was determined by measuring the activity of N-acetyl-hexosaminidase as described by Landegren (1984). Briefly, the cells were lysed in low salt buffer containing Triton X-100 and incubated at pH 5 with p-nitro-phenyl-N-acetyl-β-D-glucosamine as a substrate for the lysosomal hexosaminidase. The reaction was stopped with the addition of high pH buffer and the amount of liberated p-nitro-phenol was quantitated at 405nm.

RESULTS

Attachment of chondrocytes to matrix macromolecules after preincubation in spinner culture.

Tracheal chondrocytes were isolated by collagenase digestion and kept in spinner culture for 24 hours to allow the cells to reconstitute their cell surface. These cells were tested for ability to attach to plastic coated with a number of extracellular macromolecules, fig.1. Fibronectin and collagen II, expectedly, gave good attachment of the chondrocytes compared with plastic coated with bovine serum albumin (BSA). The 36kDa cartilage matrix protein consistently showed good attachment, equal to fibronectin and collagen II. The 58kDa-protein also supported attachment but was somewhat less efficient. The 59kDa- and the 400kDa-protein promoted some attachment, but with considerable variation between experiments. Occasionally binding to both these proteins were somewhat more marked, but the particular experiment shown is representative. Osteopontin, which is a bone specific, sialic acid rich protein containing the same cell binding sequence as fibronectin and vitronectin (Franzén & Heinegård, 1985; Oldbergetal., 1986), was also tested and it mediated attachment of chondrocytes. Neither hyaluronic acid nor large aggregating proteoglycan and small proteoglycan supported attachment. Notably also the 148 kDa protein never promoted attachment.

A considerable variation in the maximal number of cells bound was found between experiments. It varied from 49% to 16% of cells added, but was usually above 35%.

Attachment of chondrocytes directly after cell isolation

Chondrocytes used directly after isolation, fig.2a-b, differed from cells preincubated in spinner cultures by also attaching to the proteoglycans PG-LA and PG-S. Attachment to 59kDa- and 400kDa-protein was more pronounced than in cells preincubated in spinner culture, fig 2c. The cells, however, in no case attached to hyaluronic acid.

PG-LA and PG-S both contain chondroitin sulphate chains which could mediate binding. Therefore in a parallell experiment free chondroitin sulphate was included in the medium to block attachment via the chondroitin sulphate side chains of the proteoglycans, fig 2b. The added chondroitin sulphate had no substantial effect on the attachment to proteoglycans, indicating that the

promoting effect of PG-LA and PG-S probably is mediated by the protein core of the proteoglycans. Adding dextran sulphate (Pharmacia Biochemicals) at the same concentration drastically lowered attachment in all wells (not shown). After the cells from the same cell preparation had been cultured in spinner culture for 44hrs they did not attach to the proteoglycans, fig.2c.

In this particular set of experiments the 59kDa- and the 400kDa-protein showed good binding even after the incubation of the cells in spinner culture.

Attachment of other cells than tracheal chondrocytes.

Limb cartilage are of a different developmental origin than tracheal cartilage. Therefore articular cartilage chondrocytes were tested, fig. 3a. These chondrocytes showed the essentially the same pattern of attachment as tracheal chondrocytes.

Fibronectin, collagenII and osteopontin clearly promoted attachment of bovine scleral fibroblasts, fig. 3b. A problem with these cells is the high nonspecific binding observed in control wells coated with BSA. Lower values was obtained with the proteoglycans, hyaluronic acid and the 148kDa-protein, which therefore may be better as controls. Relative to those macromolecules all the other cartilage proteins were positive.

Also cells from sources other than connective tissue were studied, i.e. rat hepatocytes, fig 3c. These cells showed a very good attachment to fibronectin, 89% of the cells becoming attached. The hepatocytes interestingly also showed some attachment to osteopontin. None of the other molecules tested showed any attachment promoting effect.

Rat chondrosarcoma cells were tested to demonstrate whether species differences might be responsible for the lack of binding of rat hepatocytes to 36kDa- and 58kDa-proteins, fig 3d. The chondrosarcoma cells showed good attachment to fibronectin, 36kDa- and 58kDa-proteins but surprisingly not to osteopontin. This indicates that attachment mechanisms to fibronectin and osteopontin is not identical. It has indeed been shown that osteopontin binds to a vitronectin receptor on osteosarcoma cells (Å. Oldberg, personal communication).

Competition for attachment with peptides specific for the cell binding domain of fibronectin

An experiment was made to determine if attachment to 36kDa- and 58kDa-protein is related to the well documented mechanism for cell attachment to fibronectin. An Arg-Gly-Asp sequence located in the cell binding domain of fibronectin has been shown to be essential for attachment of cells to fibronectin. Addition of a synthetic hexapeptide containing this sequence to cell medium has been shown to block attachment of fibroblasts to fibronectin (Pierschbacher & Ruoslahti, 1984). A control peptide only differing with the Asp substituted for Glu in the binding sequence does not block attachment.

Serial dilutions of active and control peptide were used to block attachment of chondrocytes preincubated in spinner culture, fig 4. Only attachment to fibronectin and osteopontin could be blocked by the active peptide, fig. 4a,d. Both control and active peptide showed some inhibition of attachment to 36kDa- and 58kDa-proteins, fig. 4b,c.

Spreading of attached cells

Attachment can be considered as an indication of the presence of a receptor for the particular molecule to which the cells attach. Isolated hepatocytes attach without spreading to substrates coated with asialoprotein (Rubin et al., 1981). Adhesion proteins like fibronectin in addition induce a flattening and spreading of attached cells within 1-6 hours after initial attachment. Thus fibronectin is considered to mediate the binding of the cells to the extracellular matrix. To obtain an indication if any of the cartilage matrix proteins could mediate not only attachment but also spreading of chondrocytes we monitored the behaviour of chondrocytes with time in culture on the different macromolecules.

The chondrocytes spread to a much lesser extent, even on fibronectin, compared with the fibroblasts and the hepatocytes. Only a few chondrocytes had spread on fibronectin after 6 hours. No spreading was seen after 6 hours on the 36kDa- and 58kDa-cartilage proteins. Chondrocytes were therefore cultured for 20 hours on the different proteins. During this prolonged period, however, the cells have time to synthesize their own adhesion proteins or other proteins that can bind to the coated molecules and mediate spreading. Positive effects seen might therefore not be a primary effect of the coated molecule.

When isolated cells were used directly without spinner culture, fig 5, prominent spreading after 20 hours was obtained with fibronectin, collagen II, PG-LA, PG-S and osteopontin. A small number of cells spread on 59kDa- and 400kDa-protein. Hyaluronic acid efficiently prevented spreading. Cells cultured on 36kDa-, 58kDa- and 148kDa-protein showed no spreading.

If chondrocytes kept in spinner culture before spreading was tested, fig.6, the cells spread less well. In this case no cells attached to PG-LA and PG-S.

DISCUSSION

The 36kDa-protein was in all experiments as effective as fibronectin and collagen II in promoting attachment. The 36kDa-protein appears to bind chondrocytes by a mechanism different from that of fibronectin as shown by the lack of competition for cell attachment with the peptide representative for the cell binding sequence of fibronectin. The 36kDa-protein furthermore does not promote spreading of chondrocytes. Because it is not soluble in physiological buffers the specificity of binding cannot be tested by competing for binding by adding 36kDa-protein to the medium. However, since rat hepatocytes clearly do not attach to 36kDa-protein, or to any of the other cartilage proteins tested, it appears that attachment to 36kDa-protein and the other proteins tested is not just an effect of nonspecific interaction but is the result of an interaction with molecules at the cell surface that is not present on all types of cells. The 58kDa-protein isolated from cartilage also promoted attachment of chondrocytes. This protein showed the same behaviour as the 36kDa-protein, but results varied more as an effect of time in culture and between experiments.

Attachment to large and small proteoglycans was observed only directly after isolation of the cells. After preincubation of cells in spinner culture the proteoglycans did not support attachment. In this case the cells have had opportunity to build a pericellular coat, which is likely to mask interactions occurring at the cell surface. However, not all interactions are blocked by the pericellular coat since some proteins such as fibronectin and the 36kDa-protein gives equally good results before and after spinner culture.

The technique used have limitations, i.e. studying attachment to coated plastic surfaces might give false negative results. Binding sites on molecules coated to plastic may be unavailable for binding. The large aggregating proteoglycans have been shown to bind via their hyaluronic acid binding region

to hyaluronic acid at the cell surface of chondrocytes maintained in spinner culture (Sommarin & Heinegård, 1983). Therefore the cells, after spinner culture, were expected to attach to surfaces coated with these proteoglycans. The lack of attachment can be explained by the fact that coating of PG-LA to plastic is mediated via their hyaluronic acid binding region, which then may become inaccessible for binding to cell surface hyaluronic acid.

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FIGURE LEGENDS

Figure 1. *Attachment of chondrocytes.*

Tracheal cartilage chondrocytes were cultured in spinner culture for 24 hours before attachment experiment. Maximal attachment was 30% of added cells. For details see Methods section. Vertical bars indicate standard deviation (n=3).

Figure 2. *Attachment of chondrocytes at different times after isolation.*

- a) Cells directly after isolation.
 - b) Cells directly after isolation with 40 ug of chondroitin-6-sulphate/ml added to the medium.
 - c) Cells after 48 hours in spinner culture.
- Maximal attachment was in a) and b) 47%; in c) 49%.

Figure 3. *Attachment of other cells.*

- a) Bovine articular cartilage chondrocytes. Maximal attachment was 38%.
- b) Bovine sclera fibroblasts. Maximal attachment was 43%.
- c) Rat hepatocytes. Maximal attachment was 89%.
- d) Swarm rat chondrosarcoma cells. Maximal attachment was 37%.

Figure 4. *Competition for attachment of chondrocytes with peptides specific for the fibronectin cell binding region.*

- a) Fibronectin coated to plastic.
 - b) 36kDa-protein coated to plastic.
 - c) 58kDa-protein coated to plastic.
 - d) Osteopontin coated to plastic.
- Solid line - Inactive control peptide (GRGESP)
Broken line - Active peptide (GRGDSP)

Attachment is expressed as percent of maximum attachment for each protein.

Figure 5. *Spreading of tracheal chondrocytes plated directly after cell isolation.*

Directly after isolation tracheal chondrocytes were transferred to F12HB medium and plated in microtiter wells coated with the indicated macromolecules. After 20 hours of incubation the cells were photographed using a Leitz inverted microscope.

Figure 6. *Spreading of tracheal chondrocytes plated after preincubation in spinner culture.*

Isolated chondrocytes were incubated in spinner culture for 24 hours in F12HB medium. The cells were collected and transferred to new F12HB medium, plated and incubated as above.

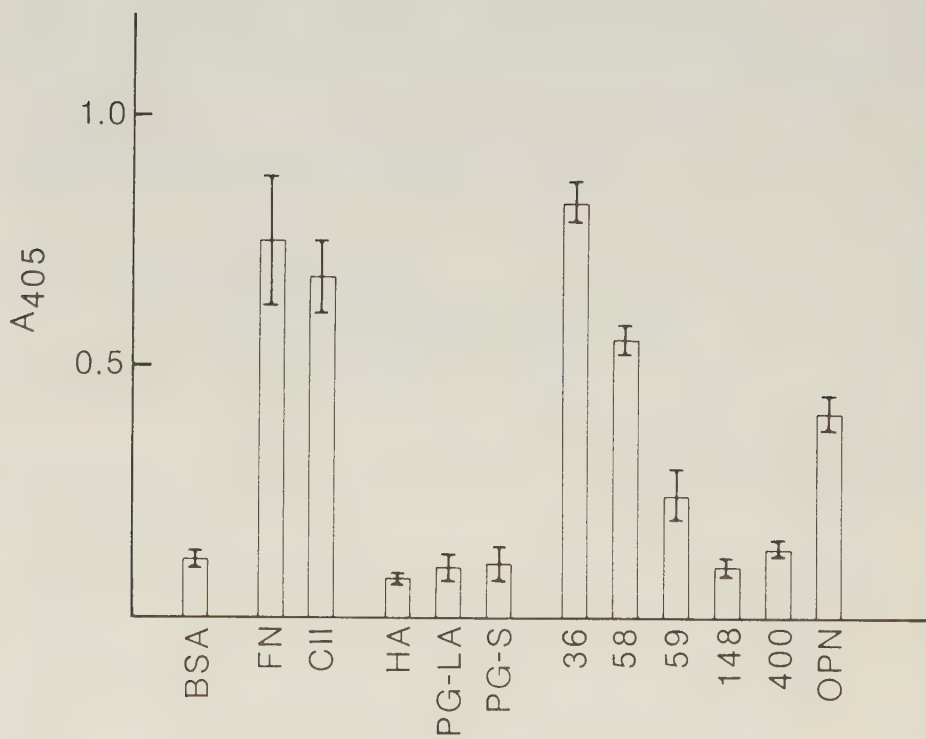


Figure 1.

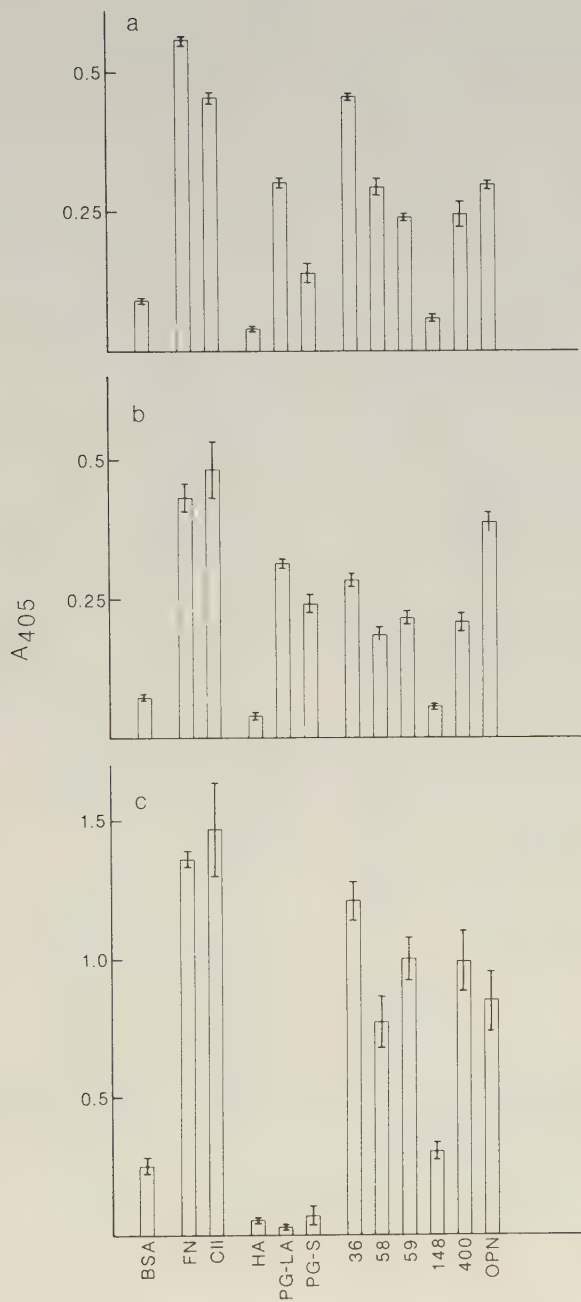


Figure 2.

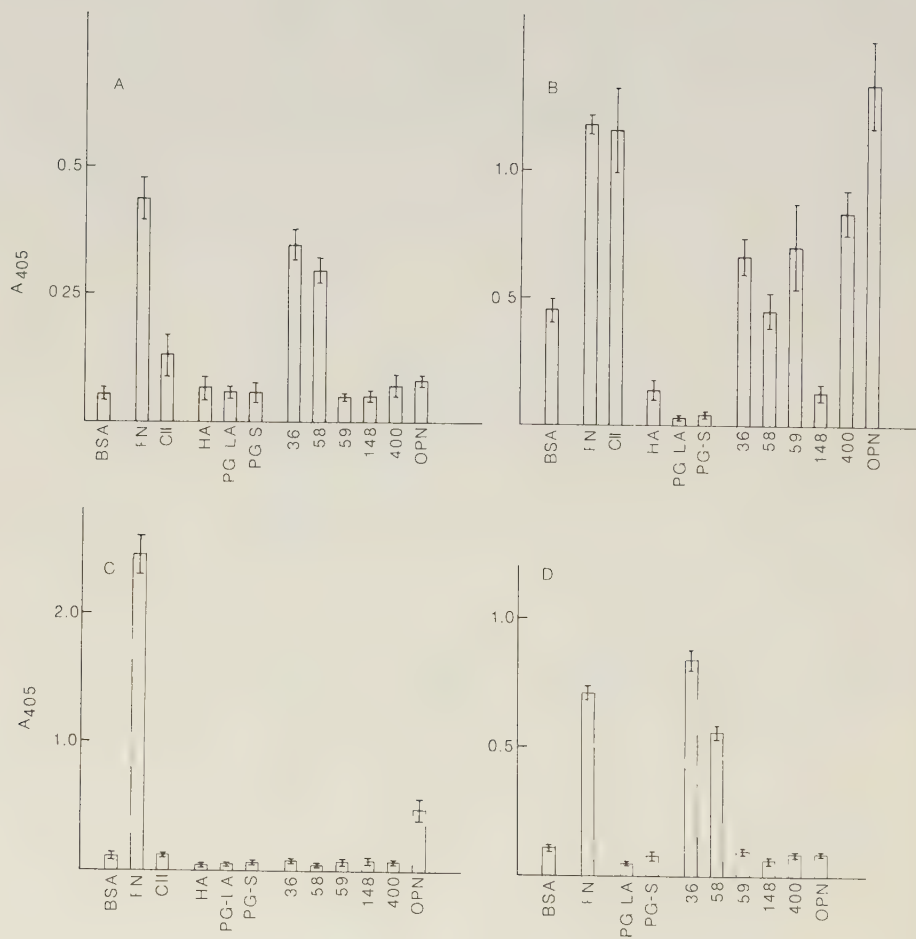


Figure 3.

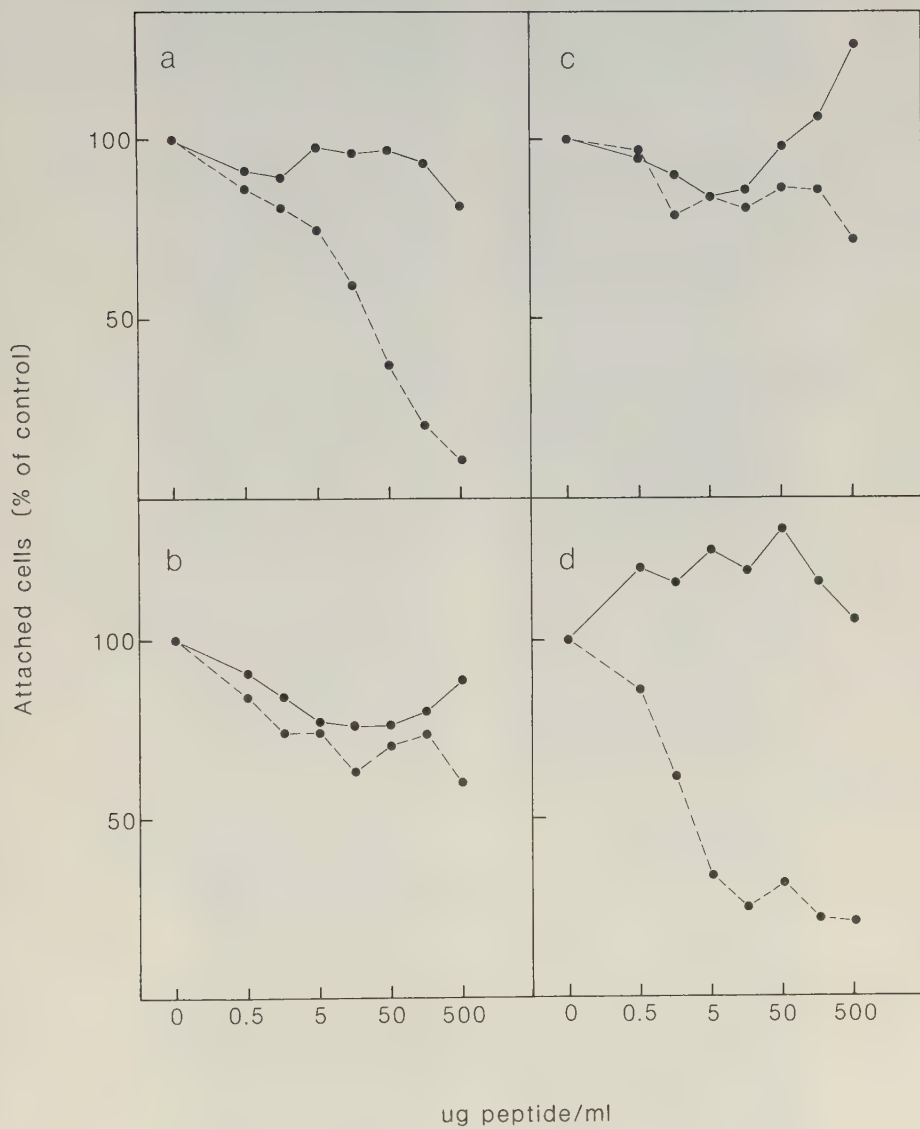


Figure 4.

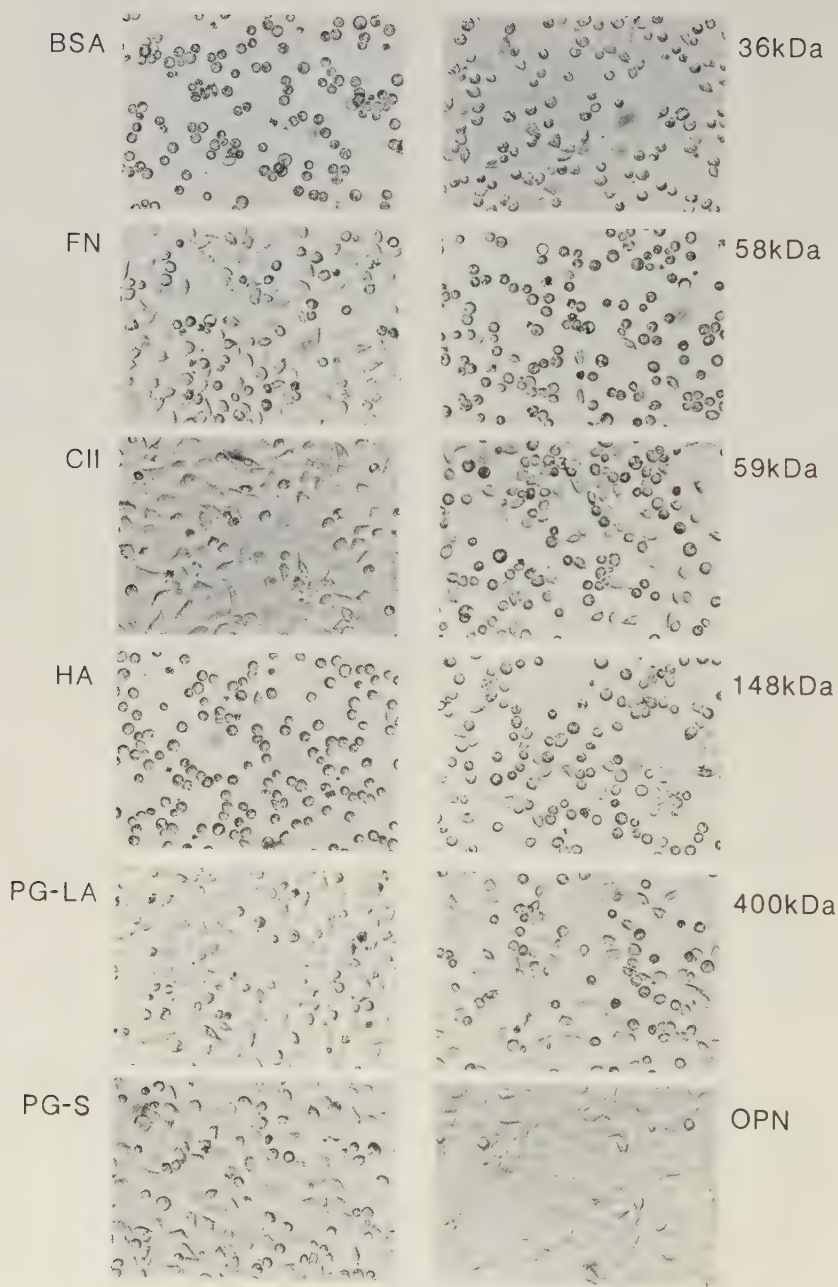


Figure 5.

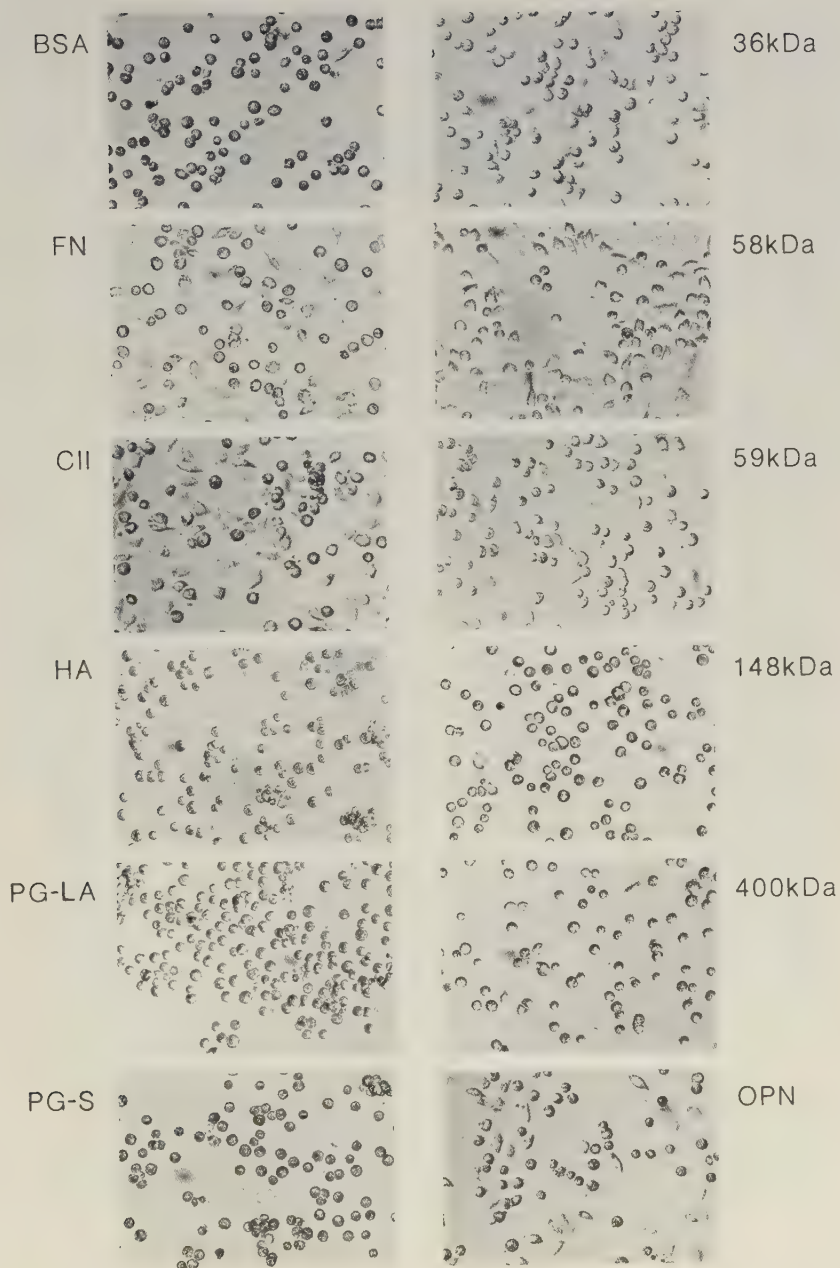


Figure 6.

Assay of Proteoglycan Populations Using Agarose-Polyacrylamide Gel Electrophoresis

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The agarose-polyacrylamide gel electrophoresis procedure for the analysis of proteoglycans originally described by C. A. McDevitt and H. Muir (1971, *Anal. Biochem.* **44**, 612-622) has been modified to minimize trailing and to allow the analysis of crude samples, i.e., tissue extracts. A slab gel system was used, permitting reproducible analysis of many samples. Procedures are described that can be used to separate and quantify several subpopulations of proteoglycans and also to quantify the proportion of proteoglycans capable of aggregating with hyaluronic acid. Applications of the procedure include transfer to nitrocellulose paper followed by immunological detection of proteoglycans as well as fluorography of separated, radiolabeled proteoglycans.

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Most tissues contain two main classes of proteoglycans (1,2). One class is composed of large molecules with molecular weights in the order of one million or more, while the other class consists of small proteoglycans with molecular weights of less than 100,000. Large proteoglycans have been identified in cartilage, tendon, aorta, and sclera, although the most typical representatives for this group are the major proteoglycans found in cartilage (1,2). They have low protein contents and contain a large number of galactosaminoglycan side chains, usually chondroitin sulfate. Most of the proteoglycans of this class appear to contain a globular hyaluronic acid binding region and can therefore interact with hyaluronic acid and form large aggregates (2). In early studies McDevitt and Muir (3) used electrophoresis in agarose-polyacrylamide gels to study cartilage proteoglycans, which are separated as two sharp bands. These two bands have recently been shown to represent two structurally distinct populations of large aggregating proteoglycans (4). Both of these proteoglycan populations are very polydisperse with respect

to molecular weight. It is therefore somewhat surprising that they do not give extremely broad bands upon electrophoresis. The mechanism for separation, therefore, appears not to depend solely on molecular weight. Cartilage also contains one other less prominent group of large proteoglycans, those nonaggregating, which cannot form aggregates with hyaluronic acid (5). Furthermore, all tissues studied, including cartilage, contain a class of small proteoglycans (1,2). These molecules have high protein contents and contain only 1-2 large galactosaminoglycan side chains of either chondroitin sulfate or dermatan sulfate. Proteoglycans of this type are found in tissues like cartilage, bone, aorta, sclera, cornea, and tendon (1). Upon electrophoresis in agarose-polyacrylamide gels, the small proteoglycans from cartilage show a higher mobility than the large proteoglycans (6).

A main disadvantage in the original technique is that it required highly purified proteoglycans for good separations and that the reproducibility was poor. In more recent work, however, Stanescu and co-workers have ob-

tained very reproducible results (for example, see (7)). Other modifications that have improved the original procedure include the use of slab gels (8). The present work describes a reproducible and improved procedure to separate and quantify the different proteoglycans. Through the use of detergents the method can be used in studies of proteoglycans in samples containing contaminating proteins. The method can also be used in the quantification of the components, either after staining or after fluorography of isotope-labeled proteoglycans. The technique may also be used to determine the capacity of proteoglycans to form aggregates with hyaluronic acid.

MATERIALS AND METHODS

Medium electroendosmosis agarose and Gelbond film were obtained from FMC Corporation, Marine Colloids Division, Rockland, Maine. Acrylamide and bisacrylamide were from Bio-Rad, Richmond, California.

Bovine cartilage samples were obtained at the local abattoir, dissected clean, and divided into small pieces, using either a Surform or a scalpel blade. Proteoglycans were extracted with 15 vol of 4 M guanidine-HCl, 0.05 M sodium acetate, pH 5.8, containing the following protease inhibitors: 0.1 M 6-amino-hexanoic acid, 50 mM benzamidinium hydrochloride, 10 mM EDTA, and 5 mM *N*-ethylmaleimide, the latter also preventing disulfide exchange (9).

Bovine nasal cartilage aggregating proteoglycans were purified as described elsewhere (4). Fractions d.D1 and d.D2 represent fractions of high and intermediate buoyant density obtained by CsCl-guanidine-hydrochloride equilibrium centrifugation of a cartilage extract (4). The fractions contain primarily chondroitin sulfate-rich and keratan sulfate-rich aggregating proteoglycans (d.D1) and keratan sulfate-rich aggregating and small proteoglycans (d.D2).

In some cases proteoglycans were precipitated from the extracts with 9 vol of ethanol as described elsewhere (10). In essence, 200 μ l of the extract was mixed with 1800 μ l of 95%

ethanol in 2-ml Eppendorf centrifuge tubes. After incubation overnight at 4°C, the sample was centrifuged at 10,000g for 15 min in a Sigma 101 centrifuge and the supernatant was removed with a syringe. When guanidine-HCl was present in the sample, the ethanol precipitation had to be repeated to ensure complete removal of the guanidine-HCl, which otherwise would form massive precipitates with the sodium dodecyl sulfate (SDS).¹ In those cases the precipitate was suspended in 100 μ l of 2.5 M sodium acetate, pH 7. Precipitation was repeated as described above.

Preparation of samples for electrophoresis. Samples, usually ethanol precipitates, were dissolved to contain 0.25 to 2 mg/ml of proteoglycan in 0.5% (w/v) SDS, depending on its nature. In general, small proteoglycans were dissolved at higher concentrations, because of their less intense staining with toluidine blue. The samples were incubated for 2 h at 37°C and prior to electrophoresis mixed with an equal volume of a solution containing 60% (w/v) sucrose, 0.05% bromphenol blue in double concentrated electrophoresis buffer (see below). The volume of the final solution applied to the gel for electrophoresis should not exceed 50 μ l, containing less than 50 μ g of small proteoglycans or less than 30 μ g of large proteoglycans. Larger amounts of proteoglycans impaired resolution and increased trailing.

When the aggregating capacity of the proteoglycans with hyaluronic acid was tested, the samples were dissolved in stock buffer (0.04 M Tris-acetate, 1 mM sodium sulfate, pH 6.8) without SDS. High-molecular-weight hyaluronic acid (Healon, Pharmacia) was added (routinely 20% by weight of the proteoglycan) and the solution was incubated overnight at 4°C. Immediately prior to electrophoresis, an equal volume of a solution containing 60% (w/v) sucrose, 0.1% (w/v) Triton X-100, and 0.05% (w/v) bromphenol blue was added.

¹ Abbreviations used: SDS, sodium dodecyl sulfate; CS, chondroitin sulfate; KS, keratan sulfate; HA, hyaluronic acid.

Casting of gels. An LKB vertical slab gel electrophoresis cell was used (equivalent cells can be obtained from Bio-Rad or Hoefer Scientific). Gels, 3 mm thick, were cast between frosted glass plates (14 × 18 cm) with a 10 place comb mounted. It was found to be imperative to use frosted plates, since the gel did not adhere properly to plain glass plates, with the result that "pockets" formed between the plate and the gel, resulting in free electrophoresis.

Acrylamide stock solution (25 ml; 9.12 g acrylamide and 0.48 g *N,N*-methylenebisacrylamide/100 ml of distilled water), Triton X-100 (175 mg), and *N,N,N',N'*-tetramethylethylenediamine (0.75 ml) was mixed in an Erlenmeyer flask (100 ml) and equilibrated at room temperature.

Agarose (1.20 g) was suspended in 112.5 ml of stock buffer (0.04 M Tris-acetate, 1 mM sodium sulfate, pH 6.8) in a 500-ml suction flask. The agarose was then dissolved by heating the flask with constant stirring, care being taken to ensure that all agarose was dissolved. Loss of water was determined by weighing and compensated by adding distilled water. The final concentrations of the cast gel were 0.6% agarose, 1.2% (T) acrylamide (with 5% cross-linking). Freshly made ammonium persulfate (0.6 g in 15 ml of distilled water) and the acrylamide solution were then rapidly added to the agarose solution before it had cooled significantly. The mixture was briefly (less than 30 s) degassed with a water aspirator and immediately poured between the plates. The amount of solution added was sufficient only to cover the bottom 3–5 mm of the combs. If a larger portion was covered, the gel cracked easily when the combs were removed. To prevent too early gelation of the agarose, it was found to be useful to preheat the plates to 37 or 45°C, although with experience this precaution became superfluous. The gels were left for 2 h at room temperature and then electrophoresis buffer (stock buffer diluted 4 times with water, i.e., 1 + 3) was added to the top of the gel to prevent drying. Electrophoresis could be done after 2–3 h, but it was found

preferable to leave the gel overnight or longer at 4°C to ensure temperature equilibration. Immediately prior to electrophoresis the comb was carefully removed. It was of help to add buffer to prevent air from entering between the comb and the gel.

Electrophoresis. Stock buffer diluted 4 times was used as electrode buffer. Electrophoresis was done with cooling at 2–4°C. The gels were preelectrophoresed for 1 h using 50 V (3–4 V/cm). Samples (20–50 μ l) were carefully layered on top of the gel, with the plates remaining in the cell and without removing the buffer from the upper reservoir. We found it convenient to apply the samples using a Hamilton syringe with a narrow, 3- to 5-cm-long Teflon tubing fitted. If critical, this tubing was easily changed between samples. The electrophoresis was done overnight (15–18 h) at 50 V. The indicator band migrated to a position about 5–8 cm down the gel during this time. Its position, however, was found not to indicate the front, but merely to indicate adequate sample handling and an effective electrophoretic run.

After electrophoresis the gels were carefully transferred to a fix solution (methanol:acetic acid:water; 50:7:43). Since the gels were brittle, it was of some help to slide the plates carefully against each other before separating one plate from the gel. Handling was somewhat facilitated if the bottom 2–3 cm of the gel was cut off. The gel was most easily pushed into the vessel containing the fix solution using one of the glass plates as a support for the gel. After 1–2 h of fixation, the proteoglycans were seen as opalescent precipitates in the gel. Staining was done using toluidine blue (0.2 g/liter in 3% v/v acetic acid). Under these acid conditions, proteins and hyaluronic acid showed no staining. After adequate destaining with 3% v/v acetic acid, the gel was transferred to the hydrophilic side of a Gelbond film. A wetted filter paper was placed on top of the gel, followed by absorbent paper, a glass plate, and a 2- to 3-kg weight. After some 20 min the layers of paper were removed and the film was dried at 60°C for 30 min. The resolution of the electrophoresis remained throughout the drying

procedure and the dry gel was easily scanned using a gel scanner.

For detection of ^3H - or ^{35}S -labeled proteoglycans, we used the fluorographic method of Chamberlain (11), with sodium salicylate as scintillator. The gel was suspended in 10 vol of distilled water for 30 min either directly after electrophoresis or after destaining. Thereafter the gel was incubated in 4 vol of 1.3 M sodium salicylate, 5 mM sodium phosphate, pH 7, for 90 min. The gel was then dried on thick filter paper using a vacuum gel drier and fluorography was done using Kodak XAR-5 X-ray film.

The proteoglycans were also electrophoretically transferred to nitrocellulose paper, using an electrophoretic transfer cell, and then stained and identified using antibodies. Handling of the very brittle gel, however, required some practice. We found it helpful to use a support of a fine nylon sieve between the gel and the backup filter.

RESULTS AND DISCUSSION

One major problem with the original technique is considerable trailing, even when pure proteoglycan samples are used. By including Triton X-100 in the gel, however, most of this trailing was abolished and good resolution was obtained. The results were similar for 0.05 and 0.1% Triton X-100.

In many cases it is desirable to analyze crude samples of proteoglycans or samples of proteoglycan aggregates. In such cases it is necessary to prevent interaction of proteoglycans with other components in the sample. This can be done in several ways. In the case of proteoglycan aggregates not stabilized by link proteins, i.e., such formed of proteoglycan monomers and hyaluronic acid, they can be dissociated by the addition of hyaluronic acid deca- or dodecasaccharide at 1/10 of the proteoglycan weight (14, data not shown). Another procedure, i.e., to incubate samples with SDS prior to electrophoresis, was originally used by Oegema *et al.* (12) with aorta proteoglycans and by Thonar *et al.* (13) with articular

cartilage proteoglycans. This type of procedure was found to work well when samples were dissolved in 0.5–1.0% (w/v) SDS prior to electrophoresis and incubated for 2 h at 37°C to allow the SDS to bind to any protein present. When these conditions were used, even samples containing large amounts of contaminating proteins could be analyzed with good resolution of proteoglycans. Thus proteoglycans in extracts of cartilage could be precipitated with ethanol dissolved in SDS and electrophoresed. With samples from tissues having a low proteoglycan content, it was usually necessary to make at least a crude purification of the proteoglycan before analysis, in order not to overload the gel by large quantities of protein.

With some batches of agarose, a spurious band seen as a precipitate after fixation was observed. This band did not stain with toluidine blue, but at times it interfered with the separation. The position of this band could be shifted away from the proteoglycans by adding Triton X-100 to the sample immediately prior to electrophoresis at 4 times excess (w/w) over SDS. Mixed micelles were thereby formed with free SDS. The Triton was alternatively included in the sucrose solution used to increase the density of sample. Interestingly, under these conditions aggregation with hyaluronic acid could occur, shifting some of the material to the slower migration of the aggregate. This was probably an effect of binding excess SDS in mixed micelles with Triton.

With all precautions taken, the proteoglycans in extracts of several cartilages could be separated after ethanol precipitation (Fig. 1). All samples had the same general appearance, with two major bands representing the two populations of aggregating proteoglycans (4), the slower migrating band representing the chondroitin sulfate-rich proteoglycan, and the faster migrating band representing the keratan sulfate-rich proteoglycan. A fast migrating, barely visible minor component representing the small proteoglycans was also seen (6). Interestingly, the pattern of proteoglycans differs between cartilages and even between samples

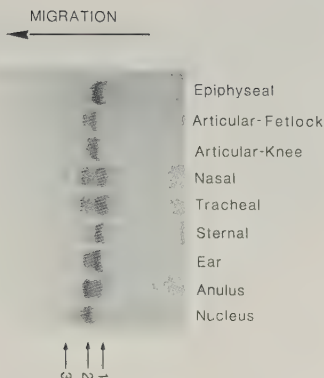


FIG. 1. Agarose-polyacrylamide gel electrophoresis of extracts of various bovine cartilages. Extracts with 4 M guanidinium chloride were precipitated with ethanol and dissolved in 0.5% SDS as described under Materials and Methods. The arrows indicate the migration positions of chondroitin sulfate-rich proteoglycans (1), keratan sulfate-rich proteoglycans (2) and small proteoglycans (3).

of bovine articular cartilage from two different joints (Fig. 1). Analogous results have been obtained with samples of purified proteoglycans from a number of cartilage sources by Stanescu and co-workers (7).

Quantification of Proteoglycans

Samples containing different amounts of proteoglycans were applied to gels, electrophoresed, fixed, stained, and dried on Gelbond films as described above.

The gels were scanned using a Zeineh soft laser scanner, but it was found that the best results were obtained when a tungsten lamp and a filter with 645-nm transmittance were used. One sample analyzed represents an extract of nasal cartilage containing a mixture of cartilage chondroitin sulfate-rich proteoglycans, keratan sulfate-rich proteoglycans (4), and small proteoglycans (9). Prior to electrophoresis, the proteoglycans were precipitated with ethanol as described under Materials and Methods. As shown in Fig. 2, all three proteo-

glycans gave linear relations between dilution and area of the scans. In another set of electrophoretic runs, a sample containing a purified mixture of keratan sulfate-rich and small proteoglycans from cartilage was electrophoresed (Fig. 3). Again, the dilution curves were linear. It should be stressed, however, that the color yield on a weight basis of each type of proteoglycan is different. Therefore, to achieve accurate quantification, it was found to be imperative to use standards of the same type of proteoglycan.

In separate experiments, it was shown that destaining of one gel in 400 + 200 ml of 3% (v/v) acetic acid for 24 h each gave absorbances upon scanning that were 2/3 of those obtained when destaining was done for only 4 h in 400 ml. In all cases, however, the dilution curves were linear (data not shown), showing that the time for destaining was not critical for quantification. A destaining time of 4 h was usually adequate and was used for those experiments shown in Figs. 2 and 3.

Analysis of Aggregating Capacity

Determination of the capacity of proteoglycans to form aggregates with hyaluronic acid

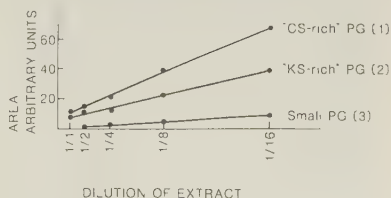


FIG. 2. Quantification of proteoglycans present in an extract of bovine nasal cartilage. The proteoglycans were precipitated with ethanol and redissolved in 0.5% SDS as described. Samples corresponding to the dilutions given were applied to agarose-polyacrylamide gels. The gels were stained with toluidine blue and destained for 4 h in 3% aqueous acetic acid. They were then dried on Gelbond films as described and scanned with a gel scanner. The areas under each peak were calculated by cutting out the peak from a copy (Xerox) and weighing the paper. The components assayed were those indicated by arrows 1, 2, and 3 in Fig. 1.

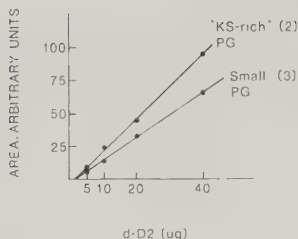


FIG. 3. Quantification of two populations of proteoglycans present in a subfraction purified by CsCl density gradient centrifugation (4). The subfraction was a fraction (a d.D2 preparation, see Ref. (4)) dialyzed against water and its proteoglycan content was determined approximately as the dry weight of the lyophilized sample. Dilutions of the stock solution were made by including SDS to a final concentration of 0.5%. If the SDS is omitted, the proteoglycans form aggregates with the hyaluronic acid present in this fraction. The proteoglycans were electrophoresed and quantified as before. The components quantified were those indicated by arrows 2 and 3 in Fig. 1.

often presents problems. For instance, proteoglycans from some sources, such as young rat cartilage, seem to bind less strongly to hyaluronic acid, and the normally used proportion of hyaluronate to proteoglycan of 1–2:100 does not give a good yield of aggregates when small amounts are analyzed (data not shown).

The electrophoretic procedure described appear to circumvent these problems by showing more efficient formation of aggregates with hyaluronate. It was therefore used to detect aggregate formation with hyaluronate. The aggregates can be identified because of their lower mobility compared with that of the monomer. Two preparations of bovine nasal cartilage proteoglycans (4) were incubated with different amounts of hyaluronic acid (no SDS present) and aggregate and monomer were quantified after electrophoresis (Fig. 4 and Table 1). In a control experiment the aggregation capacity of the samples was tested using 500- μ g samples with 2% (w/v) of hyaluronic acid analyzed on a Sepharose 2B column as described elsewhere (15).

The proportion of the large proteoglycan monomers that formed complexes with hy-

aluronic acid and was retarded upon electrophoresis was about 80% for all concentrations of hyaluronate (Table 1). This is in accordance with previously published values using other methods of detecting aggregation (2), and similar figures of 70% (d.D1) and 78% (d.D2) were obtained in control experiments with gel filtration on Sepharose 2B of hyaluronate–proteoglycan mixtures (chromatograms not shown). Aggregation was observed both with the slow migrating chondroitin sulfate-rich and with the somewhat faster migrating keratan sulfate-rich proteoglycans. The somewhat low values for aggregation of the “CS-rich” population in fraction d.D2 (Table 1) were probably due to some trailing of aggregates. The component observed at the position of these proteoglycans, i.e., the minor band in the control sample, was absent in samples with added hyaluronic acid. The small fast migrating proteoglycans were expectedly not retarded

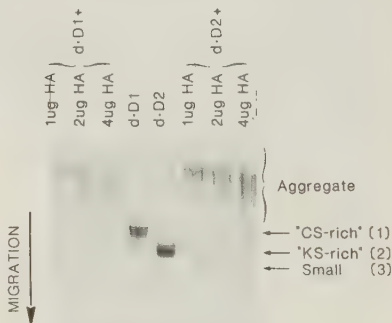


FIG. 4. Aggregation of proteoglycans by hyaluronic acid, added before electrophoresis. Samples of d.D1 (10 μ g) and d.D2 (15 μ g) (for definition of the fractions see Ref. (4)) were mixed with the indicated amounts of hyaluronic acid (HA) incubated overnight at 4°C and electrophoresed on an agarose–polyacrylamide gel. The gel was stained, dried, and scanned as described before. The positions of the two subpopulations (CS-rich and KS-rich) of aggregating proteoglycans and the small proteoglycans are shown. Scans of only aggregate (indicated) and the CS-rich and KS-rich aggregating proteoglycans were used for calculations of aggregating capacity (Table 1).

TABLE 1

AGGREGATION OF PROTEOGLYCANS WITH HYALURONIC ACID, MEASURED AS CHANGED MIGRATION AFTER ADDITION OF INCREASING PROPORTIONS OF HYALURONIC TO d.D1 PROTEOGLYCAN AND d.D2 PROTEOGLYCAN PREPARATIONS, RESPECTIVELY

	No HA	1 μ g HA	2 μ g HA	4 μ g HA
d.D1 preparation (10 μ g)				
Aggregate	0 (71) ^a	74 (73)	76 (76)	77 (78)
CS-rich	71	26 (27)	24 (24)	23 (22)
KS-rich	29			
Small	—	—	—	—
d.D2 preparation (15 μ g)				
Aggregate	0 (70)	77 (80)	82 (80)	81 (78)
CS-rich	14 (14)	6 (9)	6 (10)	8 (11)
KS-rich	86 (16)	17 (11)	12 (10)	11 (11)
Small (percentage of total proteoglycans)	8.1 (5.6)	6.7 (6)	6.1 (6.4)	7.2 (5)

Note. Percentage aggregation (indicated in Fig. 4) is calculated relative to the amount of the large proteoglycans (CS-rich plus KS-rich) only.

^a Values within parentheses obtained with 1 μ g/ml of hyaluronic acid in the gel.

after addition of hyaluronic acid (Fig. 4 and Table 1), showing their lack of a hyaluronic acid binding region (1,9).

The aggregate formation at the lowest concentration of hyaluronate was somewhat lower than with high concentrations. Therefore, a relative proportion of 20% hyaluronate to proteoglycan was chosen. Since hyaluronic acid (not stained at the acid pH chosen) migrated considerably slower than the proteoglycans upon electrophoresis (data not shown), the aggregates would tend to dissociate during electrophoresis. Therefore, in a control experiment hyaluronic acid was included in the gel (Table 1, values within parentheses). In this case any proteoglycans dissociated and separated from the aggregate would continuously be exposed to fresh hyaluronate and therefore again form aggregates. This system would be more similar to a steady-state equilibrium than most other techniques used. The results, however, were very similar to those with only hyaluronate added to the samples (Table 1), with values of aggregation from 75 to 80%. The small proteoglycans expectedly moved to the

same position as in the gels without hyaluronate, showing that aggregation was specific for certain classes of proteoglycans. Even without previous addition of hyaluronate to the samples, aggregation was almost complete. We suggest that routinely, for simplicity, the procedure with no hyaluronic acid in the gel can be used, but when samples from new sources are analyzed a test should first be made with hyaluronic acid included in the gel to optimize conditions for aggregation and allow the detection of weaker interactions. For control, reduced and alkylated proteoglycans have been electrophoresed with added hyaluronic acid. No retardation of the reduced monomers was seen, as would be expected from their inability to form specific aggregates with hyaluronate (15, data not shown).

The procedure presented can be used for the study of proteoglycans from a number of sources. In this communication, we have restricted the description to cartilage proteoglycans and to show detection of components using staining with toluidine blue. We have, however, also used the procedure to separate

radiolabeled proteoglycans from various sources, including human skin fibroblast culture medium, with detection of at least four bands of labeled molecules by fluorography (data not shown). In another application we have used electrophoretic transblotting of separated proteoglycans to nitrocellulose sheets, followed by detection with antibodies directed against proteoglycans (data not shown).

In summary, then, the electrophoretic procedure as presently described provides a useful tool in the study of proteoglycan subpopulations and their properties.

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